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THE ROLE OF CYTOKINES IN THE PATHOGENESIS OF EXPERIMENTAL *LEGIONELLA PNEUMOPHILA* INFECTIONS

BY

Corinna M. Krinos

B.S. University of New Hampshire 1994

DISSERTATION

Submitted to the University of New Hampshire

in Partial Fulfillment of

the Requirements for the Degree of

Doctor of Philosophy

in

Microbiology

May, 1999

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Ph.D. DISSERTATION

This dissertation has been examined and approved.

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TO MY MOTHER AND FATHER

THIS WORK COULD NOT HAVE BEEN COMPLETED

WITHOUT YOUR ENDLESS LOVE AND SUPPORT.

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ACKNOWLEDGEMENTS

I would like to thank the College of Life Sciences and Agriculture for a CoLSA Summer Fellowship and the Graduate School for their support in the form of research funds and teaching assistantships.

I would like to extend my deepest gratitude to my advisor Dr. Frank G. Rodgers for believing in me and offering me the invaluable opportunity to conduct this research project. I sincerely thank the members of my committee: Dr. Roger E. Wells for his help in pathology and enlightening conversations, Dr. Arthur O. Tzianabos for his support and encouragement, Dr. Andy P. Laudano for his confidence and Dr. Robert M. Zsigray for his inspiration and patience.

During my time at UNH I have experienced both hardships and joys and during those times I have meet many wonderful and inspiring people who have helped me along the way. I would like to sincerely thank Robert Mooney for his endless help in scientific and not so scientific matters. I would like to extend a deep thank you to Aubrey van Gould III for his invaluable help, support and conversations that I will treasure always. A very sincere thank you to Frank Gibson for his invaluable help, and encouragement and for his extreme patience, limitless love and faith in me. I want to deeply thank my parents for their constant support, everlasting love, encouragement and for always believing in me.

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TABLE OF CONTENTS

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LIST OF TABLES

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LIST OF FIGURES

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LIST OF PLATES

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ABSTRACT

ADHERENCE, INTRACELLULAR REPLICATION AND THE HOST CYTOKINE RESPONSE DURING *LEGIONELLA PNEUMOPHILA* INFECTION BOTH *IN VITRO* AND *IN VIVO*

by
Corinna M. Krinos
University of New Hampshire, May, 1999

Legionnaires' disease is an acute lobar pneumonia caused, primarily by the intracellular pathogen *Legionella pneumophila.* This organism when inhaled by humans descends into the lower respiratory tract and parasitizes alveolar macrophages. *L. pneumophila* adhered to U-937 cells, A549 cells and peritoneal macrophages from A/J mice in an opsonin-independent fashion. Following attachment, the organism penetrated the cell membrane, replicated within these cells eventually inducing lysis. To better define the adhesion of *L. pneumophila* to host cells, an *E. coli* clone (LP 116), expressing the 25 kDa major outer membrane protein (MOMP) of *L. pneumophila* was used in binding studies. This MOMP-expressing clone showed a 5-fold increase in opsonin-independent binding to U-937 cells as compared to the parent *E. coli* strain and suggests that the 25 kDa MOMP of *L. pneumophila* functions as an adhesin.

A major thrust of this study was to better define the cytokine response of the host during *L. pneumophila* infection. Challenge of macrophage-like

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U-937 cells with live, killed or fixed *L. pneumophila* elicited the secretion of some cytokines while downregulating others, suggesting that *L. pneumophila* modulates cytokine production *in vitro*. To develop a better understanding of the complexity of the immune response to *L. pneumophila* infection, an experimental model of Legionnaires' disease in A/J mice was developed. A/J mice were challenged intratracheally with a sublethal dose of *L. pneumophila* to investigate bacterial replication and to characterize the progression of pneumonic disease. Challenge of mice with *L. pneumophila* resulted in an 8-fold increase in the numbers of *L. pneumophila* in the lungs within 24 h followed by gradual clearing of the organism. During infection with *L. pneumophila*, gross and histopathological evaluation indicated pneumonic infection. In addition, infection with *L. pneumophila* produced both proinflammatory and immunoregulating cytokines as assayed from bronchoalveolar lavage fluids, lung homogenates and sera. This biphasic approach demonstrated that *L. pneumophila* elicits the expression of some cytokines while modifying the levels of others produced by macrophages. Furthermore, *L. pneumophila* induced a specific cytokine response in A/J mice following lung infection.

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CHAPTER I

History of *Legionella* and Legionnaires' Disease

1.1 Historical Perspective

Legionella pneumophila gained notoriety in July of 1976 at an American Legion Convention held in Philadelphia Pennsylvania at the Bellevue-Stratford hotel during the Bicentennial celebration of the Declaration of Independence. A total of 221 individuals subsequently developed an acute respiratory disease and 34 died (131). Of the 221 identified cases of disease, 182 were members of the Pennsylvania branch of the American Legion and 29 died after returning home from the convention. The 39 additional non-legionnaires who were either in or passed by the hotel also developed what was subsequently termed Broad Street pneumonia and of these 5 died (131). The source of the disease although never proven, was suspected to be a contaminated ventilation system. There followed a medical media event that played out as the weeks passed. The news media turned its attention to the mysterious disease identifying each new case and publishing hundreds of articles in newspapers and weekly magazines. Epidemiological and microbiological studies continued for 6 months. It was believed by many that the cause of the disease was chemical, viral or toxin related but a bacterial

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etiology was not suspected as pathogenic bacteria were not isolated from the victims. It was not until January of 1977 that Dr. Joseph McDade at the Centers for Disease Control and Prevention (CDC) identified the causative agent using rickettsial methodology (233). The agent was named *Legionella pneumophila* from the disease it caused (Legionnaires' disease) and from the pneumonic symptoms of those affected. Failure to initially isolate or identify the agent was caused due to a lack of staining with the typical Gram stain procedure which uses safranin, nor did it grow on traditional bacteriological media (233). That specimens had been tested for rickettsiae by inoculation into guinea pigs was fortuitous and lead to the isolation and identification of the bacterium.

Guinea pigs inoculated with lung tissue of infected individuals became ill, however, there was no transmission of disease from guinea pig to guinea pig and organisms were not isolated when subcultured on routine agar. Embryonate hens' eggs were used in an attempt to isolate pathogens; however, this technique used for the isolation of rickettsiae proved unsuccessful, probably because of the use of penicillin and streptomycin to suppress contaminants. Omitting the antibiotics and using the Giemenez stain, which was developed for *Rickettsia* and contains carbol fuchsin, gram negative bacilli were seen (233). In this way an entirely new family of pathogenic bacteria was identified. Retrospective studies have shown that the disease, although unrecognized, has occurred in epidemics since the 1940's

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(260, 333, 338).

In addition to Legionnaires' disease, the pathogen causes another, albeit milder, non-pneumonic condition that, like the pneumonic form, had occurred in explosive outbreaks in the past but had gone undiagnosed until 1978. One such unsolved episode was an acute respiratory condition that afflicted 144 employees and visitors at the Oakland County Health Department in Pontiac Michigan in 1968 (156, 198). This form of the disease was named Pontiac Fever and differed from the Philadelphia outbreak in key properties (Table 1). Legionnaires' disease is a potentially fatal multifocal necrotizing pneumonia while Pontiac Fever presents as a self-limiting flu-like illness. Although there were no fatalities in Pontiac, the attack rate was 95% (156). No definitive explanation has been offered for these two disparate disease conditions collectively referred to as legionellosis; indeed, both are caused by apparently identical genotypic and phenotypic organisms. In this respect, the role of the immune system in the host is a key factor in disease expression.

The first agar medium reported by Feeley *et al.* (116) to support sparse growth of *L. pneumophila* was Mueller-Hinton agar supplemented with 1% hemoglobin and 1% Iso Vitale X^R. Dumoff used chocolate agar to isolate *L. pneumophila* from lung tissue and pleural fluid from a patient who died from a pneumonia of previously unknown etiology (97). Feeley *et al.* (116) identified the essential growth factor present in Iso Vitale X^R as L-cysteine

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Table 1: Clinical Presentation of Legionnaires' disease versus Pontiac Fever

	Legionnaires' Disease	Pontiac Fever
Named for:	Philadelphia outbreak, 1976	Pontiac outbreak, 1968
Attack rate (%):	1-5	95
Incubation period (days):	2-10	1-2
Clinical syndrom:	Pneumonia	Non-pneumonic
Symptoms common to both:	Fever, cough, headache, confusion, chest pains, nausea, malaise, diarrhea and vomiting	
Symptoms unique to each:	Dyspnoea, haemoptysis, upper respiratory tract infection, abdominal pain, non-purulent sputum production	Pleuritic pain
Other sites affected:	Central nervous system, gastrointestinal tract, kidneys and pericardium	None
Fatality rate (%):	0-40	0

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which, together with ferric ions in hemoglobin, were required for successful isolation of the pathogen. Substituting yeast extract for casein hydrolysate, and adding activated charcoal (115), α-ketoglutarate (99) and ACES (N-2-acetamido-2-aminoethanesulfonic acid) buffer (265) improved organism growth on solid media logarithmically. This medium, buffered charcoal yeast extract (BCYE-α) agar is the mainstay of the bacteriology, clinical and research laboratory for growing this organism and substantial growth appears within 24 to 48 h incubation at 37°C in a humid atmosphere. *L. pneumophila* has a specific requirement for iron which is met by the addition of ferric pyrophosphate to BCYE-α (275). A broth medium formulated from the same components as BCYE, but lacking the agar and charcoal and which is filtered, also supports the growth of legionellae (279).

Legionella species are environmental organisms and are ubiquitous in fresh water environments. Legionellae have been isolated from lakes, rivers, mud samples, thermal lagoons and potable water supplies (130, 245, 319, 240, 127). In the environment these organisms multiply within a variety of protozoa (296, 295, 18). Legionellae parasitize and replicate within these cells and are then released into the environment from where they enter man-made water supplies. Human infection typically occurs when contaminated aerosolized water droplets are inhaled and the organisms descend into the lower respiratory tree where they primarily infect alveolar

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macrophages (180). Legionellae are not primary human disease agents, but rather are opportunistic pathogens which, after gaining entry into the host, cause severe disease.

Several investigators have studied the pathogenesis of *L. pneumophila* using *in vitro* and *in vivo* models of infection. There are a variety of human and animal cells that support the intracellular replication of *L. pneumophila*. Alveolar macrophages are the primary cells infected in humans; however, peripheral blood monocytes also support the intracellular replication of the organism (188). A variety of animal models have been investigated to study *L. pneumophila* infection and these include guinea pigs, rats, mice, monkeys and hamsters (132, 315, 263, 55). However, each animal system provides advantages and disadvantages for delineating the pathogenesis of *L. pneumophila*.

1.2 The Organism

1.2.1 Taxonomy and nomenclature. In 1979 Brenner *et al.* (54) using deoxyribonucleic acid (DNA) homology established that this newly identified organism represented not only a new genus but a new family of bacteria, the *Legionellaceae*, comprised of a single genus. Since the initial identification of *L. pneumophila*, numerous additional species have been added to the genus, which currently consists of 39 species and 3 subspecies and numerous serogroups (Table 2). Two additional genera were proposed, *Tatlockia* and

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Table 2: Taxonomy and classification of the legionellae[a]

Species	Serogroups	Source	β–lac	Ox	Hip	Gl	BP	AF	M
L. pneumophila[b]	14	H/E	+	V	+	+	+	-	+
L. adelaidensis	1	E	-	-	-	+	-	-	+
L. anisa	1	H/E	+	+	-	+	+	BW(V)	+
L. birminghamensis	1	H	+	V	-	+	-	YG	+
L. bozemanii[c]	2	H/E	V	V	-	+	+	B W	+
L. brunensis	1	E	+	-	-	+	+[e]	-	+
L. cincinnatiensis	1	H	-	+	-	+	+	-	+
L. cherrii	1	E	+	+	-	+	+	B W	+
L. dumoffii[c]	1	H/E	+	-	-	+	V	B W	+
L. erythra	2	E	+	+	-	+	+	R	+
L. fairfieldensis	1	E	-	+[e]	-	-	-	-	-
L. feelii	2	H/E	-	-	V	-	+[e]	-	+
L. geestiana	1	E	-	-	+[e]	+	+[e]	-	+
L. gormanii[c]	1	H/E	+	-	-	+	+	B W	+
L. gratiana	1	E	+	+	-	+	-	-	+
L. hackeliae	2	H	+	+	-	+	+	-	+
L. israelensis	1	H/E	+	-	-	+[e]	+	-	+
L. jamestowniensis	1	E	+	-	-	+	+	-	+
L. jordanis	1	H/E	+	+	-	+	+	-	+
L. lansinsensis	1	H	-	+	-	-	-	-	+
L. londiniensis	1	E	+	-	-/+[e]	+	+	-	-
L. longbeachae	2	H	V	+	-	+	+	-	+
L. maceachernii	1	H/E	-	+	-	+	+	-	+
L. micdadei[d]	1	H/E	-	+	-	-	-	-	+
L. moravica	1	E	+	+[e]	-	+	+[e]	-	+
L. nautarum	1	E	+	+	-	-	-	-	-
L. oakridgensis	1	H/E	+[e]	-	-	+	+	-	-
L. parisiensis	1	E	+	+	-	+	+	B W	+
L. quarteirensis	1	E	+	-	-	+	+	-	+
L. quinlivanii	2	E	-	-	-	+	+	-	+
L. rubrilucens	1	E	+	-	-	+	+	R	+
L. sainthelensi	2	H/E	+	+	-	+	+	-	+
L. santicrucis	1	E	+	+	+	+	+	-	+
L. shakespearei	1	E	-	+[e]	-	+	-	-	+
L. spiritensis	2	E	+	+	+[e]	+	+	-	+
L. steigerwaltii	1	E	+	-	-	+	+	B W	+
L. tucsonensis	1	H	+	-	-	+	-	B W	+
L. wadsworthii	1	H	+	-	-	+	-	-	+
L. worsleiensis	1	E	+	-	-	+	+	-	+

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Table adapted from Rodgers and Pasculle (1991)
[a]All strains are catalase positive (*L. hackliae, L. londinensis and L. worsleiensis* are weakly positive) and all fail to reduce nitrate.
[b]*L. pneumophila* contains three subspecies: *L. pneumophila* subspecies *pneumophila,* subspecies *pascullei* and subspecies *fraseri.*
[c]Alternate genus name : *Fluoribacter*
[d]Alternate genus name: *Tatlockia*
[e]Some strains are weakly positive only

+ = positive reaction; - = negative reaction; **V** = variable results

β-lac = β-lactamase; **Ox** = oxidase; **Hip** = hipurate; **Gl** = gelatin liquification; **BP** = brown pigment; **AF** = autofluorescence; **BW** = blue/white fluorescence; **YG** = yellow/green fluorescence; **R** = red fluorescence; **M**= motility

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Fluoribacter, to encompass *L. micdadei* and the autofluorescent species *L. bozemanii*, *L. dumoffii* and *L. gormanii* respectively (146). However, there has not been universal acceptance of this nomenclature system. Serological reactions, determination of cellular fatty acids, and isoprenoid quinone analysis are useful for classification; however, these do not differentiate all species, thus DNA relatedness remains the mainstay of classification (51). Presumptive identification of *Legionellae* is accomplished by growth on BCYE-α with L-cysteine and α-ketoglutarate and the absence of growth on blood agar or BCYE-α without L-cysteine with subsequent confirmation using a variety of bacteriological and molecular techniques (51).

1.2.2 **Physico-chemical properties.** *L. pneumophila* organisms grow optimally on complex bacteriological media at temperatures between 35-37°C and at an optimum pH of 6.9 with a narrow range between 6.85-6.95 for clinical isolates. The use of potassium buffers rather than those that contain sodium are preferable as sodium ions can restrict or inhibit *Legionella* growth (116). It has been suggested that the purpose of charcoal and α-ketoglutarate in the medium is to detoxify BCYE-α by neutralizing the toxic activity of free fatty acids such as, oleic acid produced in the media during autoclave sterilization. Alternatively, charcoal may scavage toxic oxygen radicals released from the yeast extract present in the medium especially after

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exposure to bright light (176). In the environment, *Legionella* has been isolated from thermal lagoons and cooling towers at temperatures in excess of 60°C and generally grow well over a wide temperature range (127).

1.2.3 **Cultural and morphological characteristics.** Although legionellae are facultative intracellular organisms they are readily cultured on bacteriological media and colonies appear in 1-3 days. Typically, colonies are opalescent, resemble cut glass and have a stringy consistency. During early growth, colonies show a pink or blue-green iridescence which disappears with prolonged incubation at which time large and small colony variants develop. These appear grey-white in color, losing their characteristic appearance as they become more opaque resembling other bacteria. Some *Legionella* species autofluoresce brilliant blue-white whereas *L. pneumophila* does not (54, 213). Yet others autofluoresce either yellow-green or red (Table 2) (116, 234).

The morphological characteristics of *L. pneumophila* are shown in Table 3. *L. pneumophila* is a gram negative, fastidious, pleiomorphic rod ranging in size from 0.3 to 0.9 μm in width by 2 to 20 μm or more in length (289, 283, 68, 51). When grown on bacteriological media *L.pneumophila* appear as elongated or filamentous rods up to 50 μm in length with tapered ends; however, when viewed from autopsied lung, rods are seen as short coccobacilli (68, 283, 290). *L. pneumophila* is a motile, non-sporeforming, aerobic, non-acid fast, non-encapsulated bacterium. Legionellae exhibit

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Table 3: Morphological characteristics of *L. pneumophila*

- Slender gram negative rods (*in vivo* forms are short 0.5 x 1-2 μm)
  (from bacteriological media 0.4 x 2-3 μm)
- Pleiomorphic (long froms can reach 20 μm in length *in vivo* and > 50 μm in length *in vitro*)
- Divide by non-septate, pinching, binary fission
- Non-parallel sides with tapered ends
- Internal PHB storage granules
- Vacuolated surfaces, convoluted or smooth surfaces
- Possess lipopolysaccharide (F-1 antigen) on bacterial surface
- Motile by polar or sub-polar flagella
- Fimbriae present
- No evidence of acid mucopolysaccharide capsule
- Blebs on outer membrane surface
- Outer membrane and cytoplasmic membrane each 10 nm thick separated by a thin peptidoglycan layer
- Ribosomes 25 nm in diameter

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cellular architecture typical of gram-negative bacteria when examined by electron microscopy (289, 283). The double layer cell envelope of the organism is approximately 25 nm thick and comprises the periplasm located between the outer and cytoplasmic membranes each measuring approximately 10 nm in width (289, 283, 284). Peptidoglycan and diaminopimelic acid, a component of peptidoglycan as well as 2-keto-3-deoxyoctonate (KDO), a component of lipopolysaccharide (LPS), have been identified in *L. pneumophila* (126, 283, 284). The LPS of legionellae is smooth and the species and serologic differences in composition allow for serogroup differentiation (142, 166, 254). *L. pneumophila* LPS consists of a membrane-associated lipid portion that is rich in branched-chain fatty acids and is tightly associated with the major outer membrane protein (MOMP). In addition, *L. pneumophila*, possesses a polysaccharide complex comprised of glucose, rhamnose, mannose, quinovosamine, KDO and glucosamine. The O-antigenic side chain is responsible for the characteristic ladder-like banding appearance in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels as well as for the serogroup specificity detected in sera (167, 318). Patients elicit strong antigenic responses to the LPS of the organism. Poly-β-hydrohybutyrate (PHB) storage granules have been identified as electron-lucent vacuoles measuring 30 to 200 nm (33). However, larger forms up to 0.5 μm have been identified by negative stain, thin section and freeze-fracture electron microscopy (289, 283, 284, 281). James *et. al.* (193) has

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recently described that *L. pneumophila* accumulate intracellular reserves of PHB, which support its long-term survival in a culturable state under starvation conditions. It was suggested that this endogenous energy reserve is likely to play an important role in promoting the persistence of legionellae in stressful low-nutrient environments outside the amoebal host. The majority of species, except for *L. oakridgensis*, *L. nautarum* and *L. londiniensis* are motile by means of polar or sub-polar flagella (284, 287) (Table 2) and these are unsheathed measuring 14-20 nm in diameter and up to 8 μm in length (287, 288). The individual monomeric flagellin proteins have a molecular mass of 47 kDa, as determined by SDS-PAGE (262). The expression of flagella is nutrient and temperature dependent (262). In addition, the legionellae were shown by electron microscopy to possess pili (287) and this finding has recently been confirmed by genetic techniques. Ribosomes measuring 25 nm in diameter are found in the cytoplasm along with the fine skein of nuclear material typical of gram-negative bacteria (289, 283, 287).

1.2.4 Biochemical properties. *L. pneumophila* possesses the enzymes catalase, peroxidase, gelatinase, phospholipase and β-lactamase; they are oxidase variable and have the ability to hydrolyze hippurate (Table 2) (339, 54, 80, 168). Under longwave ultraviolet light (366 nm) some species display blue-white or red fluorescence that may assist in species identification (54, 80). They contain high levels of branched chain fatty acids in their cell wall (51);

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however, the presence of an extracellular acid polysaccharide layer detectable by ruthenium red staining has remained in doubt (169, 283). The organisms are nutritionally fastidious, utilizing amino acids such as arginine, threonine, methionine, serine, valine, and cysteine as energy sources rather than carbohydrates (355, 268, 334, 337, 335, 150). The organism in particular has an unusually high requirement for L-cysteine (116, 268). The organic acids; lactate, pyruvate, acetate, malate, fumarate, and oxaloacetate serve to stimulate oxygen consumption by these bacteria.

Even though *L. pneumophila* can hydrolyze starch and oxidize certain sugars, its energy metabolism is based on oxidation-dependent rather than fermentative pathways (116, 268, 355). *L. pneumophila* primarily derives its energy from the oxidation of amino acids by utilizing the Krebs cycle and a complex electron transport chain, that is expressed in these organisms (150, 337). Hoffman and Pine (175) have detected the presence of cytochromes of the c, b, a, and d types. The slow metabolism of glucose by *L. pneumophila* is used for biosynthesis rather than as an energy source (356, 337). Most of the glucose that is consumed by the organism is metabolized and broken down by the Entner-Doudoroff and pentose phosphate pathway, presumably to provide pentoses for nucleic acid synthesis and NADPH for the reducing power that the organism needs for various biosynthetic pathways, such as fatty acid biosynthesis (337). The Embden-Meyerhof pathway does not function in glucose catabolism, however, the gluconeogenic anabolic

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enzymes of this pathway are responsible for sugar synthesis (150, 175, 356).

Most branched chain fatty acids are synthesized by *L. pneumophila*, the predominant one being isopalmitate, a saturated, branched, 16-carbon fatty acid (244). *L. pneumophila* also contains hydroxy fatty acids, such as β-hydroxyisomyristic acid, β-hydroxyarachidic acid which are mostly confined to cell wall structures (232). The major phospholipids of *L. pneumophila* are phosphatidylcholine, cardiolipin (diphosphatidylglycerol), phosphatidylethanolamine, phosphatidylglycerol and phosphatidyldimethylethanolamine (124).

When examined, the strains of *L. pneumophila* all produced extracellular protease, phosphatase, lipase, DNase, RNase, and β-lactamase activity. They did not however, produce elastase, collagenase, hyaluronidase, chondroitinase, neuraminidase, urease or coagulase (340).

Important ions for *Legionella* cultivation, include calcium, cobalt, copper, magnesium, manganese, molybdenum, nickel, potassium, vanadium and zinc (275, 336). Legionellae show a requirement for iron and although the organism will grow at iron concentrations of 0.5 μm, excess amounts are usually needed for optimal growth of the organism and for the efficient operation of bacterial enzymes, ferredoxins and cytochromes. Iron is also involved in the bacterial process of electron transport, regulation of gene expression and oxygen metabolism.

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1.2.5 **Environmental aspects.** The *Legionellaceae* are environmental organisms that are ubiquitous in fresh water environments. Legionellae have been isolated from a wide range of fresh water habitats, and in particular those that have been thermally polluted, including lakes, rivers, ground water, thermal lagoons and potable water supplies (130, 245, 45, 49). Isolates have also been obtained from moist potting soils, mud, river banks and rain water run-off (319). The association of *L. pneumophila* with blue-green algae and fresh water amoebae in the aquatic environment is well documented (341, 28, 296, 18). The organism multiplies intracellularly in a variety of protozoa belonging to the genera *Hartmanella* (121, 56), *Acanthamoeba* (6, 316), *Naegleria* (252), *Echinamoeba, Tetrahymena* (122) and *Cyclidium* (49, 18, 344) (Table 4). *Legionella* parasitize and replicate within these protozoa and are then released into the environment. It is from these sources that legionellae gain entry into man-made water supplies such as water systems of large buildings, evaporative condensers and cooling towers (4, 79, 92, 138, 145). *L. pneumophila* have been isolated from aerosols created by many sources such as evaporative air conditioners, decorative fountains (117), whirlpool baths, showers (45, 49), humidifiers (221), supermarket vegetable water misting systems (219) and respiratory care equipment (13, 229). When *L. pneumophila* comes into contact with humans infection typically occurs provided the organisms are presented in proper fashion. As aerosolized water droplets of approximately 5-15 μm in diameter are inhaled,

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Table 4: Protozoa supporting the growth of legionellae

Category	Organism
Amoeba	*Acanthamoeba castellanii*
	A. polyphaga
	A. palestinensis
	A. royreba
	A. culbertsoni
	Naegleria gruberi
	N. fowleri
	N. lovaniensis
	N. jadini
	Hartmanella vermiformis
	H. cantabrigiensis
	Vahlkampfia jugosa
	Echinamoeba exudans
Ciliated protozoan	*Tetrahymena pyriformis*
	T. vorax

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the organisms evade the constitutive barriers of the upper respiratory tract by virtue of the droplet size and descend into the lower respiratory tree where they primarily infect alveolar macrophages eventually leading to the disease state. It has been suggested that the presence of cyanobacteria, flavobacteria, secretions of certain aquatic plants, algae and free-living amoebae may serve to either stabilize and protect the organisms in aerosols or to actively stimulate their growth (353, 352, 351, 320). Cirillo *et al.* (76) demonstrated that *L. pneumophila* grown in *Acanthamoeba castellanii* were more invasive for epithelial cells and macrophages than were *L. pneumophila* grown on agar. Replication of *L. pneumophila* in protozoans present in domestic water supplies may be necessary to produce bacteria that are competent to enter mammalian cells and produce human disease. Invasion and intracellular replication within protozoa are thought to be major factors in the transmission of Legionnaires' disease. Legionellae are protected within protozoal cysts which show increased resistance to drying and to water treatment processes such as chlorination and heating. Furthermore, bacterial load may be increased in aerosols containing these protozoa (341, 351). Thus, *Legionella* is delivered in an amplified and protected manner to the human host. It is possible that the mechanisms involved in the recognition, survival and replication of the pathogen within protozoa may be reflected in the target cell during Legionnaires' disease; namely the human alveolar macrophage. Similar interaction phenomena occur in both cell types in which *Legionella*

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are taken-up, replicate intracellularly within similar endocytic compartments and are released (2). Recently a model for attachment and entry of *L. pneumophila* into *Hartmanella vermiformis* has been proposed (348, 347). Venkataraman *et al.* (348, 347 reported the identification of a 170-kD galactose/N- acetyl-D-galactosamine (Gal/GalNAc) lectin in the protozoan host, *H. vermiformis,* as a potential receptor for attachment and invasion by *L. pneumophila* . It was demonstrated that attachment of *L. pneumophila* to the *H. vermiformis* 170-kD lectin was required for invasion and was associated with tyrosine dephosphorylation of the Gal/GalNAc lectin in addition to other host proteins (348). However, Hard *et al.* (162) provided biochemical and genetic evidence that the mechanisms of attachment and subsequent uptake of *L. pneumophila* by *H. vermiformis* and *Acanthamoeba polyphaga* were, in part, different, yet invasion and subsequent killing of the host cell was followed by similar intracellular replication within a rough endoplasmic reticulum-surrounded phagosome (162). Many of the mechanisms utilized by *L. pneumophila* to parasitize mammalian and protozoan cells are suggested to be similar, however, Gao *et al.* (144) using transposon insertion mutagenesis of *L. pneumophila* to detect mutants that exhibited defective phenotypes of cytopathogenicity and intracellular replication within macrophage-like U937 cells but not within *A. polyphaga* demonstrated that there were unique mechanisms utilized by *L. pneumophila* to survive and replicate within macrophages but not protozoa.

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In addition, unique genetic loci were required for *L. pneumophila* to parasitize mammalian but not protozoan cells. These data suggested that *L. pneumophila* has evolved as a protozoan parasite in the environment but has acquired loci specific for intracellular replication within macrophages. Alternatively, ecological coevolution with protozoa has allowed *L. pneumophila* to possess multiple redundant mechanisms to parasitize protozoa and that some of these mechanisms do not function within macrophages (144). It has been suggested that *Legionella* may recognize a highly conserved structure on the surface of the alveolar macrophage which structurally mimics a similar molecule on amoebae, however, this hypothesis has not been confirmed. Legionellae are important medical pathogens, yet, the human host is a dead end infection as there is no mechanism for return to the environment or to another suitable host. Human to human spread of the disease has never been documented.

1.2.6 Genetics. The legionellae possess a genome size of 2.5×10^9 daltons and a guanine plus cytosine (G + C) content in their DNA of 38-52 mol % (54, 104). DNA homology studies were used to determine that the organism constituted not only a new genus, but also a new bacterial family (54). Based on DNA homology and 16S ribosomal studies, the legionellae are distinguishable from and unrelated to other bacterial genera (139, 302). DNA-hybridization assay remains the recognized method for species identification.

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Commercial DNA probes have been developed to identify *Legionella* species and strains in diagnostic and environmental settings (99, 98, 319, 342). DNA relatedness is high (up to 99 %) between strains and serogroups for individual species, but ranges from 1 to 67 % between different species within the genus (52). *L. pneumophila* was divided into three subspecies, (*pneumophila, pascullei* and *fraseri*), based on a much reduced DNA homology of two of the new subspecies to the type strain Philadelphia 1 (53, 308). In addition to divergence in DNA homology, differences in enzyme profiles and amino acid requirements were reported for these subspecies.

Plasmids ranging in size from 21 to 85 MDa have been reported from both clinical and environmental strains and serogroups (60, 83, 204, 220, 235, 255). The presence of such plasmids has not been associated with either enhanced or reduced virulence. The presence or absence of plasmids do not modify antimicrobial resistance patterns nor change expression of virulence traits. The detection of plasmids has proved useful for the molecular differentiation of isolates for epidemiological purposes. The introduction of DNA into *L. pneumophila* has been achieved by a variety of methods which include transformation using electroporation, conjugation and transduction (70, 95, 107, 225, 349). Cloning genes for *L. pneumophila* antigens into *Escherichia coli* has been successful and valuable for characterizing virulence genes. Through such genetic manipulation, the *mip* gene encoding the 24 kDa macrophage infectivity potentiator (Mip) protein was identified as a

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virulence factor (109, 108). Mip is a surface protein of *L. pneumophila* and other *Legionella* species essential for intracellular infection (74, 73). Other genes have also been successfully cloned and expressed in *E.coli* and these include the gene responsible for the major outer membrane protein (MOMP) (170), a 38 kDa zinc metalloprotease (272) and a 19 kDa outer membrane peptidoglycan-associated protein (171, 218). Several highly conserved genes have also been cloned into *E. coli* including the *recA* gene, involved in repair of DNA (94, 374) and the *groEL* gene, responsible for production of a 60 kDa heat-shock protein (174). Other genes that have been identified include the *proA* (273) and *Ily* genes (365) responsible for the expression of the bacterial 38 kDa protease and 39 kDa hemolysin and their possible role in virulence has been suggested. The sequences of several *Legionella* genes have been reported. In some cases, the sequences showed significant homology to cognate genes from other bacteria. For example, the *Legionella recA* gene was found to be approximately 70 and 75 % similar to the *E. coli* and *Pseudomonas aeruginosa* genes, respectively (374). In addition, the sequence of the *L. pneumophila* protease revealed that the enzyme was more than 70 % homologous with *Pseudomonas* elastase (32). The specific mutation of a designated gene in its native background is the most direct and definitive approach to address the role of any specific gene in pathogenesis. To date, only three specific genetic loci have been identified in this fashion and these include the *efa, mip* and *msp* loci. An insertion in *efa*, a locus of unknown

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function, demonstrated that the methods for insertion mutagenesis per se do not cause a defect in cellular infection (72). However, a mutation in *mip* resulted in attenuated virulence in macrophages and in guinea pigs (74, 73). Interestingly, a mutation in the gene encoding the extracellular protease (*msp*) suggested that the protease has no necessary function in pathogenesis (330, 44). New genetic approaches are being implemented to discover new genes that may be important for virulence. One such method involves transfer of a genomic library to a nonreverting, avirulent *L. pneumophila* mutant. Host cells are then infected in order to select an isolate carrying a cosmid (or plasmid) that complements the mutation. A mutation that fails to evade phagosome-lysosome fusion was complemented by using this approach (222). Another approach is to focus on those genes that are expressed in the intracellular environment but not in bacteriological medium-grown bacteria. Finally, alkaline phosphatase gene fusions are being used to identify insertion mutations in genes expressing secreted proteins by using the procedure known as shuttle mutagenesis in which the transposition step is performed in the more permissive *E. coli* background (107).

As *L. pneumophila* is an intracellular pathogen able to grow within alveolar macrophages residing in a phagosome that does not fuse with lysosomes, much research has centered on defining those genes and gene products responsible for limiting interactions between the phagosomal

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compartment and lysosomes in addition to those genes necessary for intracellular survival of *L. pneumophila.* A large number of *L. pneumophila* genes, called *dot* (defect in organelle trafficking) and *icm* (intracellular multiplication), have been identified that are required for intracellular growth within human macrophages (48, 223, 271). This gene family is implicated in several aspects of virulence and appears to constitute components of a conjugal transfer system that has been adopted to prevent phagosome-lysosome fusion in the host cell and to mediate host cytotoxicity by pore formation (306). It has recently been shown that the *dot/icm* genes code for a large putative membrane complex that forms a type IV secretion system used to alter the endocytic pathway (350). The *dotA* locus from *L. pneumophila* has been identified as an essential gene for intracellular growth (29). Phagosomes formed by *dotA* mutants do not mature into a replicative vacuole and eventually fuse with lysosomes (29). Genetic and biochemical data have demonstrated that DotA is an integral inner membrane protein. Recently Roy *et al.* (297) have demonstrated that the *dotA* product is required to regulate trafficking of the *L. pneumophila* phagosome. In addition, the *icm*-dependent conjugal ability of *L. pneumophila* and its relationship to intracellular multiplication and host cell killing have been investigated. *L. pneumophila* has been shown to mediate plasmid DNA transfer and this process is dependent on several *icm* genes. *L. pneumophila* contain a functional *icm* complex that can mediate conjugal DNA transfer and is

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necessary for intracellular replication of the organism (307).

1.3 Clinical and epidemiological findings

1.3.1 Clinical presentation. Two clinical syndromes have been associated with infection by *L. pneumophila*; Legionnaires' disease, an acute febrile pneumonia and Pontiac fever, a non-pneumonic, influenza-like illness, collectively referred to as legionellosis (Table 1). The organisms responsible for Legionnaires' disease and Pontiac fever are phenotypically and genotypically indistinguishable and it is believed that the status of the host's immune system at the time of infection is crucial to the establishment and progression of the two disease states. Unlike Pontiac fever, Legionnaires' disease, afflicts those with underlying disease such as lymphoma, pulmonary distress, congestive heart failure and acquired immune deficiency syndrome (AIDS) (17). Legionnaires' disease is a multifocal necrotizing pneumonia with multisystem involvement (113). The incubation period is 2-10 days and the prodromal symptoms include acute fever, chills, headache, anorexia, rigors, myalgia and malaise (131). As infection proceeds respiratory manifestations include dyspnea, bradycardia, rales and a non-productive cough with little or no sputum production (16, 131, 333). If sputum is produced it is typically non-purulent. Absence of sputum or its lack of purulence has been suggested to be characteristic of Legionnaires' disease. The acute nature of the illness results in the need for hospitalization. As

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disease progresses lung examination reveals abnormalities with rales and very often lung consolidation being evident as lung function becomes compromised. Lung infection may be rapid and may be responsible for increasing dyspnea and acute respiratory failure. Major systematic changes also ensue including abnormal liver function tests, including elevations in creatine levels, blood gas and electrolyte imbalances as well as hyponatremia, hematuria, proteinuria and pancytopenia (85, 131, 201, 237). Extrapulmonary symptoms are common including disorientation and confusion out of proportion to the degree of fever. Central nervous system dysfunction, involvement of the heart, spleen, bone marrow, brain and lymph nodes as well as cellulitis have been documented (12, 354). In addition, gastrointestinal complaints of nausea and diarrhea occur in many patients. Acute renal failure, disseminated intravascular coagulation, shock, respiratory insufficiency, coma and circulatory collapse are the major factors leading to death (259). Bacteremia has been documented and may explain the extrapulmonary manifestations of Legionnaires' disease that include cerebral microglial granuloma, cutaneous and hepatic abscess formation, endocarditis, pericarditis and pyelonephritis (278, 280, 324).

1.3.2 **Pathology.** Radiological findings indicate that the pneumonia is lobar in nature, initially limited to one or several foci, especially in immunocompromised patients (364). The pneumonia is bilateral in 60 % of

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the cases and as the infiltrates extend into adjacent and non-adjacent areas, consolidation is evident in one or more lobes of the lung (110, 200). Pleural effusions can be detected in 25 % to 50 % of cases, however, these are usually small and may be due to underlying cardiac failure (200). Radiological changes may be extensive and slow to resolve even following recovery (35). During severe disease, diffuse multilobar involvement of the lung occurs with focal or lobar consolidation evident as either red or grey hepatization. Distinct macroscopic lesions are produced by *L. pneumophila* and are similar for all pathogenic species of *Legionella* (36, 364). These lesions may be difficult to distinguish from classical pneumococcal pneumonia. Tissue damage in the lungs is exacerbated by the production of numerous potent exotoxins and enzymes and these facilitate coagulative necrosis, congestion, hemorrhage and abscess formation and also act to suppress phagocytosis and interfere with host cell oxidative metabolism. Abscesses are rarely seen by radiologic examination, however, abscess formation has frequently been seen in tissue specimens of lungs from fatal cases of Legionnaires' disease (5, 364).

Pathological examination of infected lung tissues exhibit a severe inflammatory response indicative of acute fibrino-purulent pneumonia. During microscopic evaluation, alveoli and terminal bronchioles are distended and contain proteinaceous fibrin-rich debris in which macrophages and neutrophil polymorphonuclear leukocytes are present in varying number and proportions. The proximal bronchioles and bronchi are not

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usually involved. Alveolar damage with hyaline membranes and desquamation of lining cells has been observed in lung biopsies (256, 362).

1.3.3 Pathogenesis and virulence factors. *L. pneumophila* is a facultative intracellular bacterium that replicates within alveolar macrophages during human disease (250). Establishment of disease during infection with *L. pneumophila* depends on ill-defined virulence factors, the dose of infecting organisms and the status of the host's immune system. The precise mechanisms by which *L. pneumophila* infects host cells thus leading to human disease are not well understood. In 1978, Rodgers *et al.* (289) first demonstrated the presence of *L. pneumophila* within lung cells by electron microscopy. To better elucidate the infectious process, particularly at the cellular level, various primary and transformed cells in addition to several animal models have been developed (267, 363, 370). Those host cells that have been used in pathogenesis studies include transformed and primary cells of epithelial, fibroblast, polymorphonuclear and mononuclear lineage from both human and animal origin (Table 5) (155, 188, 224, 258, 286). As alveolar macrophages are the primary cell infected in disease, these form the most appropriate cell type for studying cellular infection. However, these cells are not readily available therefore, macrophages or macrophage-like cells have been used to study adherence, intracellular replication and lysis of *L. pneumophila.* Among these, the most frequently used include human

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Table 5: Cells supporting *L. pneumophila* replication

- Alveolar macrophages
  (human and rodent)
- Human peripheral blood monocytes
- MRC-5 cells (human embryonic lung fibroblasts)
- Hep-2 (human epithelial laryngeal carcinoma)
- HeLa (human cervical carcinoma)
- HL-60 (human leukemia)
- Vero (african green monkey kidney)
- U-937 (human histiocytic lymphoma)

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peripheral blood monocytes and the U-937 and HL-60 cell lines. The U-937 cell has been extensively used in studies of attachment and intracellular replication of *Legionella* (153, 188, 224, 267, 286). These cells possess Fc and complement receptors at levels consistent with human alveolar macrophages (294). In addition, the intracellular replication kinetics of *L. pneumophila* within U-937 cells is similar to that in alveolar macrophages, thus making this cell line an appropriate model for cellular infection (267). *L. pneumophila* is taken up by polymorphonuclear leukocytes (PMNLs); however, these cells do not support replication of the organism (186). Epithelial and fibroblast cells which do not possess the same membrane receptors as those found on macrophages also support the cellular infectious cycle of the organism, indicating that alternative mechanisms of cellular infection exist. There are four stages of cellular infection common to intracellular pathogens; attachment of the organism to host cells, uptake by the cell and, once inside, intracellular replication begins with the terminal event being lysis of the host cell with subsequent release of the organism to allow the cycle to begin once again. Adherence of *L. pneumophila* to host cells followed by phagocytic uptake and intracellular replication are central to establishment of disease. Adherence is a necessary prelude to infection (24) and two mechanisms of attachment have been demonstrated for *L. pneumophila;* opsonin-dependent and opsonin-independent systems and each have been shown to be important in cellular infection *in vitro* (155, 186).

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In the complement-mediated system, complement receptors CR1 (CD35) and CR3 (CD11b and CD18) on human phagocytes which recognize complement fragments C3b and C3bi respectively, mediate phagocytosis of *L. pneumophila* (26). The major outer membrane protein (MOMP) on the surface of *Legionella* fixes C3 exclusively via the alternative pathway of complement activation (26). The MOMP/C3bi complex binds to CR3 on human phagocytes. The opsonin-independent mechanism of uptake occurs in an environment devoid of complement or specific antibody (155, 286). This mechanism has been shown to produce a normal bacterial replicative cycle in U-937 cells as well as in MRC-5, HEp-2 and Vero cells (Table 5).

Uptake during complement-dependent processes is often mediated by coiling phagocytosis in which long phagocyte pseudopods coil around the organism as it is internalized (182). Once phagocytosed *L. pneumophila* induce the formation of a unique phagosome that is studded with host cell ribosomes (179, 258). Formation of the vacuole entails a complex sequence of cytoplasmic events that take place during the first 4-8 h after infection and involve host cell smooth vesicles, mitochondria and ribosomes. The significance of these observations has not been delineated, however, these processes may be important for the survival of the organism within host cells. A variety of mechanisms have been implicated in the intracellular survival of different bacteria, including extraphagosomal location, resistance to oxidative and nonoxidative killing mechanisms, inhibition of

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phagosome-lysosome fusion, and interruption of phagocyte activation and the subsequent production of bactericidal oxygen metabolites (123)(317). The initial investigations of phagocytes that ingest *Legionella* species did not detect any of the variety of mechanisms used by other organisms to evade intracellular destruction. It had been demonstrated that *L. pneumophila* and *L. micdadei* were phagocytozed and remained within the phagosome. In addition, both species were susceptible to killing in vitro by H_2O_2 and other bactericidal oxygen metabolites produced by phagocytic cells. *L. pneumophila* internalization by macrophages does result in an oxidative burst, however, following ingestion of the organism, the phagosome does not fuse with host cell lysosomes, thus, the bacteria-containing phagosome does not become acidified to the low pH levels characteristic of phagosome-lysosome fusion events and which are necessary for bacterial killing (181, 185). The mechanism by which *L. pneumophila* inhibits phagosome-lysosome fusion is not well understood. Recently, Vogel *et al.* (350) have shown that the *dot/icm* genes code for a large putative membrane complex that forms a type IV secretion system used to alter the endocytic pathway and is required for *L. pneumophila* intracellular survival. Thus, *L. pneumophila* is able to circumvent the normal destructive processes of the macrophages and not only survive but replicate within these very cells "designed" by evolution to destroy foreign antigens descending into the lungs. Following unabated intracellular replication, *L. pneumophila* is released from the confinements

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of the macrophage phagosome and escapes into the extracellular milieu where progeny organisms infect other cells in the surrounding tissues thereby facilitating the continuation of disease. The mechanisms by which *L. pneumophila* is released from the phagosome and the host cell cytoplasmic membrane is not defined. However, recent evidence indicates that the release of the organism may be preceded by macrophage apoptosis (143, 246). The major secretory protein of *Legionella*, a zinc metalloprotease with cytotoxic properties may play a role in this process; however, this protease is not essential for intracellular growth or cell killing (330) and is not a required virulence factor in a guinea pig model of Legionnaires' disease (44).

Virulence in the legionellae appears to be a multifactorial process and several potential virulence factors have been described for *L. pneumophila* (Table 6) that may promote cell invasion or intracellular survival and multiplication. Mip, a 24 kDa, cell surface protein is necessary for the efficient invasion of macrophages and for full virulence in a guinea pig model of legionellosis (74, 73). *L. pneumophila mip* (Mip^-) mutants are defective in their ability to infect macrophages and to parasitize amoebae, ciliates and lung epithelial cells (74, 75). Furthermore, the phenotype of the *mip* mutant within macrophages and protozoa suggests that Mip is involved in bacterial resistance to intracellular killing (75). The role of other bacterial products in virulence is less clear. It has been demonstrated that *L. pneumophila* responds during infection of macrophages by alteration of gene expression.

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Table 6: Virulence factors from *L.pneumophila*

Virulence factor	Molecular mass (kDa)	Gene	Function
Macrophage infectivity potentiator (Mip)	24	*mip*	Macrophage internalization protein; inhibits protein kinase C
Major cytoplasmic membrane protein	58	*htpAB*	Genus common; heat-shock protein; illicits humoral response
Major outer membrane protein (MOMP)	24-29	*ompM*	Genus and species specific epitope; involved in phagocytosis; binds complement C3; associated with LPS; porin; putative adhesin
Zinc metalloprotease (MSP)	38	*proA*	Tissue and cell damaging protein
Flagella (flagellin)	47	*flaA*	Motility; common to serogroups 1-3
Hemolysin/legiolysin	39	*lly*	Hemolysis
Peptidoglycan-associated Lipoprotein (PAL)	19	*pal* (*plpAB*)	Immunogenic ND*
Phospholipase C	50-54	ND*	Hydrolyses phosphotidylcholine
Lipopolysaccharide (LPS)	ND*	ND*	Activates complement cascade; immunogenic; weak endotoxin

*ND = Not determined

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Upon infection of macrophages, the expression of at least 70 proteins was shown to be induced, 39 of which were not detected in organisms grown in defined bacteriological media (210). The expression of these newly synthesized proteins may be required for the survival of *L. pneumophila* in host cells. The response of the organisms to the intracellular environment is complex and may involve multiple regulons. Similar gene regulation events have been observed in other intracellular pathogens, such as *Salmonella* Typhimurium, which responds to the intracellular environment of macrophages by inducing the synthesis of 136 de novo proteins, some of which are well-established virulence factors (61). The mechanisms used by pathogenic bacteria to cause infection and disease usually include an interactive group of virulence determinants, sometimes coregulated, which are suited for the interaction of a particular microorganism with a specific host. Because pathogens must overcome similar host barriers, common themes in microbial pathogenesis have evolved. It may be that some of these induced proteins of legionellae and salmonellae have similar functions that allow these organisms to survive in the macrophage environment.

Extracellular enzymes, such as proteases and hemolysins have been suggested as factors that may be responsible for some of the pulmonary and extrapulmonary manifestations of legionellosis. *L. pneumophila* has been shown to produce various toxins, including legiolysin (a hemolysin), endotoxin (LPS) and a tissue destructive protease (23, 78, 142, 365).

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L. pneumophila produces a 68 kDa phosphatase which has been implicated in modulating the respiratory burst of phagocytes by inhibiting superoxide production by human PMNLs (340). However, the phosphatase does not fully account for the capacity of the bacterium to block the respiratory burst of phagocytic cells following stimulation. The phosphatase is heat sensitive and loses both phosphatase activity and its inhibitory effect on stimulated neutrophil O_2^- production after heating; however, ingestion of heated *L. micdadei* inhibits subsequent neutrophil and monocyte activation.

Baine *et al.* (15) identified that *L. pneumophila* possess a phospholipase C that is released into the medium in which the organisms are grown and hydrolyses phosphatidylcholine to produce diacylglycerol and phosphorylcholine. These investigators suggested a possible pathophysiological implication of the phospholipase C in that phosphatidylcholine is a major component of pulmonary surfactant and its destruction could impair pulmonary gas exchange. Supporting this are the alveolar hyaline membranes that have been described in *Legionella* pneumonia (362). In addition, since phosphatidylcholine is an important constituent of eukaryotic membranes, the cytolytic action of the phospholipase C might injure both inflammatory cells and lung tissue cells. There is, however, no direct evidence that the *Legionella* phospholipase C is cytolytic or affects eukaryotic cell membranes.

Legionellae posses a number of proteolytic enzymes and

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aminopeptidases, some of which remain bound to the organism and some of which are secreted into the extracellular culture medium (27). The major secretory protein of *L. pneumophila*, a zinc metalloprotease of 38 to 42 kDa, has tissue-destructive and cytotoxic properties, inhibits human phagocyte function, is an immunostimulatory molecule and can degrade proteins that may be significant to host defense mechanisms (23, 96, 42, 41, 272). *L. pneumophila* possesses both caseinase and gelatinase activities and has extensive amino acid identity and shares a similar molecular mechanism of proteolysis with the *Pseudomonas aeruginosa* elastase (32). However, its precise role in pathogenesis during Legionnaires' disease has not been elucidated. Studies using a protease-deficient mutant of *L. pneumophila* have suggested that the protease is not necessary for virulence. Despite this evidence it has been suggested that since the mutant does produce small quantities of the protease, it is possible that enough enzyme is produced *in vivo* to account for the pathology seen during disease (330). Furthermore, the mutant and its parent strain require large aerosol doses to cause death, thus, the lung damage observed may be caused by other factors that may not be significant following challenge with lower doses of bacteria more typical of experimental Legionnaires' disease (42).

L. pneumophila produces LPS which has been shown to activate the classical complement cascade and possesses endotoxic activity; however, there is contradictory evidence as to the precise role of LPS in virulence. LPS is the

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major immunogenic structure on the surface of *L. pneumophila* and greater than 98% of antibody produced by patients with Legionnaires' disease is directed towards the LPS (142). LPS is also an activator of immune modulating molecules such as interleukin-1β (IL-1β), IL-2, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) from mouse spleen cultures (39, 38). The presence of these cytokine molecules are important for the duration and regulation of the immune system.

All *Legionella* species and serogroups examined express a 58 to 60 kDa heat shock protein (Hsp60) which contains a genus-specific epitope recognized by monoclonal antibodies, as well as epitopes which are cross-reactive with many species of gram-negative bacteria. The gene encoding this protein has been cloned in *E. coli* and its complete nucleotide sequence determined (299). The protein demonstrates considerable homology to the *Mycobacterium tuberculosis* 65 kDa heat-shock protein, the GroEL protein of *E. coli*, and *Coxiella burnetii* HtbB (211, 299). The protein is highly immunogenic and is the predominant *Legionella* protein reactive with human convalescent-phase serum from patients with confirmed cases of legionellosis (40). Hoffman *et al.* (177) demonstrated that virulent, but not avirulent, strains of *L. pneumophila* respond to environmental changes associated with becoming intracellular. The response is characterized by a rapid increase in the synthesis of Hsp60 and a concomitant shift in location of Hsp60 from the cytoplasm and periplasm to the cell surface. Since Hsp60 is a molecular

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chaperone, it was suggested that it may interfere with phagosome membrane proteins involved in initiating phagolysosomal fusion (177). Although there is no evidence establishing the *Legionella* heat-shock protein as a direct virulence factor, it has been suggested that this protein may be important during the unfavorable environment inside the phagosome.

Outer membrane proteins are of interest as they come into primary contact with the inflammatory cells and immune system of the host. The major outer membrane protein (MOMP) of *L. pneumophila* is tightly associated with the LPS of *L. pneumophila* and shows a molecular mass between 24 to 29 kDa depending on the procedure used for isolation (63, 172). The MOMP protein aggregates through the formation of interchain disulfide bridges (63). Gabay *et al.* (141) demonstrated that the MOMP of *L. pneumophila* is a cation-selective porin. Porins are a class of bacterial proteins capable of inserting themselves into membranes, including those of host cells, and opening up channels through which ions can pass. Horwitz *et al.* (182) has speculated that one mechanism by which *L. pneumophila* might inhibit acidification of the monocyte phagosome is by insertion of a proton ionophore, namely, the cation-selective MOMP porin, into the monocyte membrane. Butler *et al.* (63) has suggested that the MOMP in its native and functional form when embedded into the membrane exists as a 95 kDa tetrameric complex. The MOMP selectively fixes complement component C3; however, the precise role of the MOMP in the pathogenesis of *L.*

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pneumophila remains unresolved to date, however, work performed by High, Torosian and Rodgers (170), demonstrated that when a 750 base pair DNA fragment from the virulent *L. pneumophila* strain was transformed into *E. coli*, the resulting clones expressed the *Legionella* MOMP and had increased virulence in chicken embryos compared with the parent *E. coli* strain. Further study elucidating the function in pathogenesis of the MOMP is of paramount importance since C3 is the major opsonin for the phagocytosis of *L. pneumophila* and modification of the MOMP might lead to decreased internalization of the bacterium and subsequent failure to establish disease.

The intracellular multiplication of *L. pneumophila* is iron-dependent, thus it requires between 3 to 13 μmoles of iron for minimal growth and up to 20 μmoles for maximum growth compared to 0.3 to 1.6 μmoles for other organisms. Legionellae do not possess siderophores (276). *L. pneumophila* acquires iron from the host cell where it is derived from iron-transferrin via transferrin receptors, iron-lactoferrin via lactoferrin receptors and the iron storage protein ferritin (65, 66, 64). Proteins on the organism bind lactoferrin and transferrin and reduce the iron stores of ferritin from Fe^{+++} to Fe^{++} by utilizing an iron reductase (195). The iron incorporated into *L. pneumophila* is found in seven major iron-containing proteins, one of which is an iron superoxide dismutase. The major iron-containing protein has an apparent molecular mass of 85 to 90 kDa under denaturing conditions and 210 kDa in a

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nondenaturing environment (236). Inhibition of iron by three different sources have been demonstrated and this effect inhibits *L. pneumophila* intracellular replication. First, the nonphysiologic and physiologic iron chelators, deferoxamine and apolactoferrin (65, 66). Second, the weak bases chloroquine and ammonium chloride reduce the iron pool by blocking the pH-dependent release of iron from endocytized iron-transferrin and the pH-dependent proteolysis and release of iron from iron-lactoferrin and ferritin (64). Third, IFN-γ reduces iron availability by down-regulating transferrin receptor expression and intracellular ferritin concentration (65).

1.3.4 Epidemiology. *L. pneumophila* and other members of the *Legionellaceae* are nearly ubiquitous in fresh water environments including lakes and ponds. However, in these natural water sources, legionellae are present in low concentrations. Amoebae appear to play a critical role in the amplification process of *L. pneumophila* (18, 119, 296). Biofilms, common in plumbing systems, also contribute in supporting the growth of many bacteria including *L. pneumophila* (291). Aquatic biofilms, which are widespread not only in nature but also in medical and dental devices, can be the source of serious nosocomial infections. Barbeau *et al.* (19) demonstrated that in these microbial communities, pathogens such as nontuberculous mycobacteria, *Pseudomonas aeruginosa* and *L. pneumophila* not only survive but proliferate in biofilms. The mode of transmission of legionellosis is

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inhalation of *Legionella*-contaminated aerosolized water droplets of respirable size (5-15 μm). From an epidemiological perspective, the major sources of *Legionella* contamination include air-conditioning evaporative condensers, cooling towers, humidifiers, nebulizers, showerheads, whirlpool spas, decorative fountains and vegetable misting machines (4, 45, 49, 117, 79, 92, 138, 145).

Males between the ages of 40-70 are more likely to acquire Legionnaires' disease than are females while infections in children are rare. Predisposing factors for disease include increased age, heavy smoking, previous lung disorders, high alcohol intake and diabetes. Individuals who are immunocompromised due to chemotherapy or chronic illness such as lymphomas, leukemia and AIDS are also at greater risk (33, 90, 226).

Legionella species cause 1 to 5 % of all cases of diagnosed pneumonia, although rates as high as 30 % have been reported (113). Data from a large, ongoing, population-based study of community-acquired pneumonia requiring hospitalization indicate that the annual rate of non-outbreak-related, community-acquired *Legionella* infection is 6.1/100,000 population per year (227). Extrapolation of the rate from this study conducted in Ohio to the adult population in the United States is problematic as the incidence of infection may vary by region. However, the rate translates to an estimated 11,000 cases of community-acquired *Legionella*-associated pneumonia requiring hospitalization yearly in the United States (227). This

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figure is an underestimate of the actual incidence of legionellosis as a substantial proportion of patients may not be hospitalized; indeed, some patients infected with *Legionella* species may not have pneumonia and serology, urinary antigen detection and culture are not 100 % sensitive.

Legionnaires' disease shows a world-wide incidence and can occur as epidemics, sporadic cases and nosocomial infections. Epidemic legionellosis has been reported in many countries including the United States, Great Britain, Spain, Australia, The Netherlands, Germany, Sweden, France, Canada and Italy. The incidence of disease is higher in the summer months of July, August and September. The incidence of Pontiac fever is poorly understood and difficult to assess as it presents as a self-limiting influenza-like illness and usually goes undiagnosed. Each year 1000-1300 cases of Legionnaires' disease are reported to the CDC in the United States (Figures 1.1 and 2.1). This however, may reflect a major underestimate in the incidence of disease. Although the organism was identified in 1977, many cases go unrecognized because *Legionella* is only detectable on special media that are not regularly used for the screening and diagnosis of pneumonia agents. Estimates of the incidence of legionellosis have ranged from 10,000 to 100,000 cases annually in the United States (184). *L. pneumophila* serogroup 1 is responsible for 85 % of all reported cases of community-acquired legionellosis while *L. micdadei* causes approximately 6 % (362).

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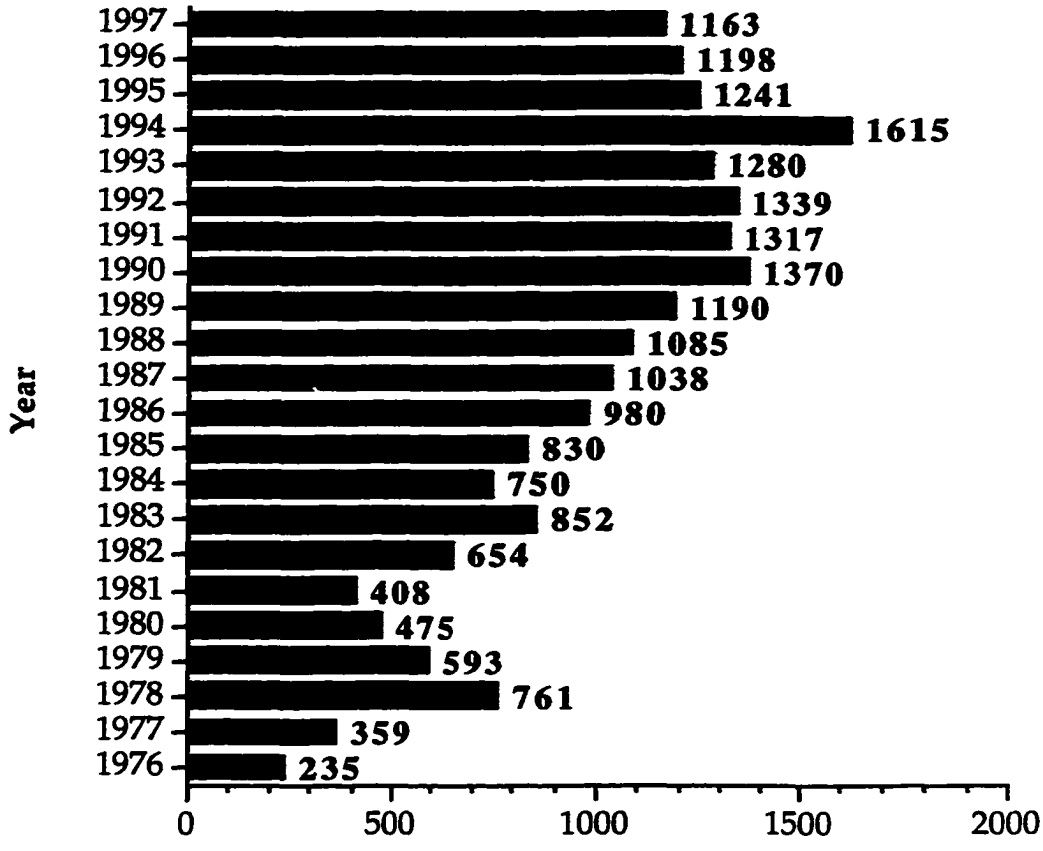

Figure 1.1: Total number of cases of legionellosis as reported by the Centers for Disease Control and Prevention (CDC) Atlanta, GA.. Adapted from C.D.C. Morbidity and Mortality Weekly Reports, Summary of Notifiable Diseases, United States 1997.

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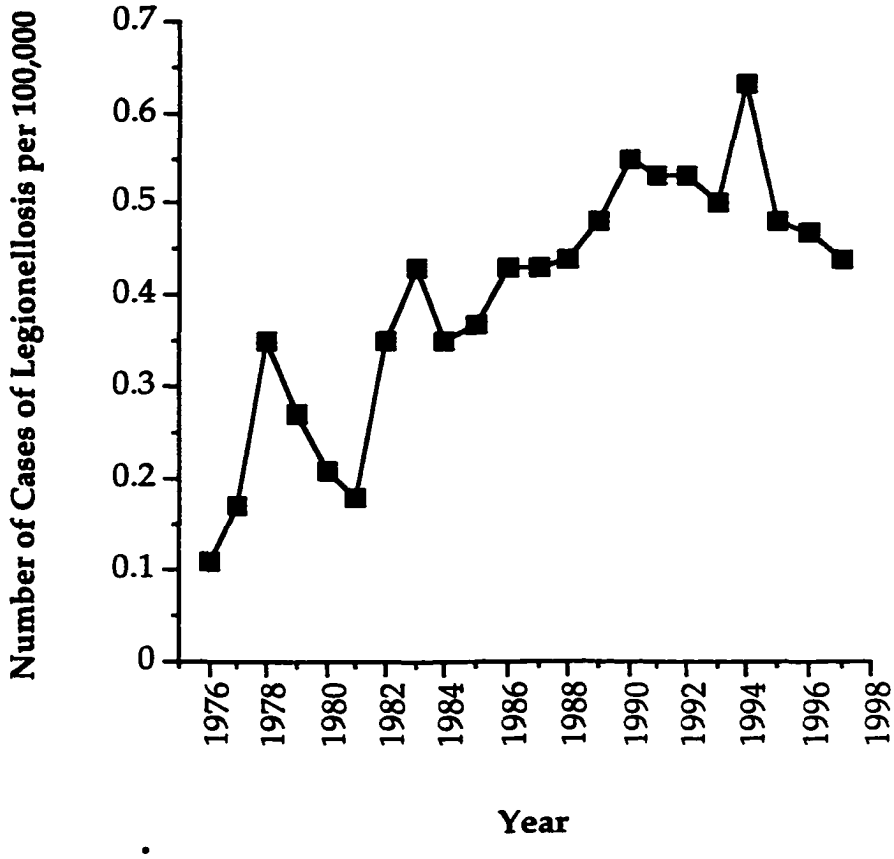

Figure 1.2: Total number of cases of legionellosis per 100,000 as reported by each state as documented by Centers for Disease Control and Prevention (CDC) Atlanta, GA.. Adapted from C.D.C. Morbidity and Mortality Weekly Reports, Summary of Notifiable Diseases, United States 1997.

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1.3.5 Diagnosis. *L. pneumophila* is a pneumonic pathogen exhibiting clinical symptoms similar to other pneumonic agents, thus making clinical diagnosis difficult. Failure to stain with traditional gram stain techniques and difficulties in growing the organism pose a problem for proper diagnosis of *Legionella* pneumonia. The mainstay of diagnosis in determining the presence of *Legionella*, is culture in that organisms grown on BCYE-α agar supplemented with L-cysteine but not on unsupplemented blood agar are presumptive for legionellae. Sensitivity of culture is between 50-80% but the specificity is 100 % (99, 360, 375). Selective media have been developed for the cultivation of *Legionella* organisms from clinical specimens that are likely to contain other microorganisms. These include BCYE-α agar with added cefamandole, polymyxin B and anisomycin (99) or alternatively glycine, vancomycin, polymyxin B and anisomycin (351). These media reduce the numbers of contaminants that would otherwise quickly overgrow the slow growing legionellae. Infection can be diagnosed by serologic means using both direct or indirect immunofluorescence to detect antibody in patient sera (100, 114, 237). Immunoassays use a specific monoclonal or polyclonal antibody to probe for the presence of bacterial soluble antigens in specimens. *Legionella* soluble antigens present in respiratory tract secretions, sputum, serum and especially in urine have been detected by enzyme-linked immunoassays, radioimmunoassays and latex agglutination and microagglutination tests (207, 206, 212, 301, 300, 357). Commercially available

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urinary antigen kits as both enzyme and radioimmunoassays exist (160). Nuclei acid probes have been developed to detect *L. pneumophila* in clinical and environmental samples (300). A ^{125}I- labelled cDNA probe is commercially available and hybridizes with the highly conserved 16S rRNA from *Legionella* and the resultant RNA-DNA duplexes are assessed by a γ-counter. The polymerase chain reaction has been used for the detection of *Legionella* antigens by adding the *mip* gene and the gene encoding 5S rRNA to *Legionella* DNA probes to improve their sensitivity (25)(219).

1.3.6 Treatment. As legionellae are facultative intracellular pathogens the effectiveness of antibiotics relies on the membrane transportability of the antibiotic in question. Only those antibiotics that possess high lipid solubility and are able to penetrate host cell membranes to gain access to the pathogen within the cytoplasm of infected target cells are effective. For this reason, macrolides have proven the most efficacious in treating Legionnaires' disease. The most widely used macrolide is erythromycin (106, 163). However, this antibiotic is not well tolerated by many individuals and results in peripheral phlebitis and intestinal distress. Recently, novel macrolide compounds in the ketolide class (semisynthetic derivatives of erythromycin) have been shown to be effective against *L. pneumophila* both *in vitro* and *in vivo* (103). Other clinically effective antibiotics include rifampin, doxycycline, co-trimoxazole and members of the 5-fluoroquinolones, such as ciprofloxacin

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(217, 290). Tzianabos and Rodgers (345) have extensively investigated the *in vitro* effects of ciprofloxacin and demonstrated that this antibiotic was effective against intracellular bacteria. In contrast, many microbial agents, including the β-lactams and aminoglycosides have no role in the treatment of *Legionella* infections (14, 201). In the absence of effective antimicrobial therapy, prognosis is poor. Contrary to *in vivo* situations, *Legionella* isolates are susceptible to a plethora of antibiotics *in vitro* (101, 228, 285).

1.3.7 Immunity and the immune response. Legionnaires' disease is more common and more severe when cell-mediated immunity is impaired, particularly in the setting of corticosteroid therapy and in individuals who either have an immunodeficiency or are immunocompromised due to drugs used for transplants or because of other co-existing conditions that affect the immune system such as that associated with AIDS (33, 90, 157, 226). During the last decade there has been considerable research defining some of the underlying mechanisms involved in the immune response to *L. pneumophila.* Cell-mediated immunity rather than humoral immunity is suggested to play a central role in host defense mechanisms against *L. pneumophila* as is the case with other facultative intracellular organisms such as *Mycobacteria, Leishmania* and *Listeria* (135, 178, 362). The development of a cell-mediated immune response leads to activation of macrophages to resist intracellular pathogens and is likely an essential host

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response to these organisms. Although the effector mechanisms of cell-mediated immunity to *L. pneumophila* in the lung have not been established, *in vitro* studies have shown that lymphokine-activated monocytes and macrophages inhibit the intracellular growth of *L. pneumophila* (31, 194, 250, 249). It has been suggested that T cell-mediated immunity begins to operate early during *L. pneumophila* infection in guinea pigs and that protective immune responses to *L. pneumophila* involve T cell-mediated delayed-type immune responses (50, 253). Cell-mediated immunity based on lymphocytes reacting with the organisms and cytokines produced by such lymphocytes are important in resistance. Vaccines prepared from killed *Legionella* or their components readily induce cell-mediated immunity (137). However, patients who recover from *Legionella* infections do develop antibody of both the IgG and IgM class. Antibody titers increase rapidly after exposure of individuals to these bacteria either by infection or immunization (137). Antibody promotes the phagocytosis of the bacteria by monocytes, alveolar macrophages and PMNLs, but does not promote complement-mediated killing of *L. pneumophila in vitro* (136). Thus, antibody produced against *Legionella* may not be protective and the net result is more bacteria gain access to the intracellular environment in which they multiply. Antibody-coated bacteria multiply at the same rate as bacteria phagocytosed in the absence of antibody.

To investigate the role of cell-mediated immunity in host defense

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against *L. pneumophila*, Horwitz *et al.* (178) demonstrated that human monocytes activated by incubation with concanavalin A and human lymphocytes inhibited the intracellular multiplication of *L. pneumophila*. In addition, monocytes activated by incubation with cell-free filtered supernatant from concanavalin A-sensitized mononuclear cell cultures also inhibited the intracellular growth of *L. pneumophila* (178). This inhibition occurred in two ways, first, the monocytes phagocytozed fewer bacteria and they slowed the rate of intracellular multiplication of bacteria that were internalized. These findings demonstrated that human monocytes could be activated to inhibit the replication of *L. pneumophila*. Thus, inhibition of *L. pneumophila* was accomplished by activating the monocytes and not by coating the bacteria with antibody and complement.

Lymphocyte sensitization to *L. pneumophila* antigen has been demonstrated in humans and laboratory animals recovering from legionellosis, and lymphokine-activated mononuclear phagocytes inhibit the replication of *L. pneumophila in vitro*. Several lymphokines are able to activate macrophages such that they exert an enhanced antimicrobial effect. IFN-γ is one of the major macrophage activating factors produced by lymphocytes for induction of anti-*Legionella* activity (38, 203, 249, 369). In addition, TNF-α has been shown to promote the anti-*Legionella* activity of neutrophils (38). It has been shown that IFN-γ treatment of murine

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macrophages collected after infection with the protozoa *Leishmania* (164, 165), *Trypanosoma* (147), *Toxoplasma* (148, 158) and *Schistosoma* (148) as well as the bacteria, *Listeria* (251)and *Mycobacteria* (373), enhances antimicrobial activities for these pathogens. These cytokines and others play a crucial role in modulating the immune response of many facultative intracellular bacteria, including *M. tuberculosis, L. monocytogenes, Brucella abortus* and *S.* Typhimurium (373, 197, 238, 251, 343, 196, 251).

In light of the high mortality rate associated with Legionnaires' disease, especially in immunocompromised individuals and the shortcoming of antibiotic therapy, alternative therapeutic strategies are being sought. Such alternates includes vaccination, immunotherapy and cytokine therapy to augment the current antimicrobial treatments available. One such avenue involves the use of naturally occurring host molecules or response systems, specifically cytokines, to amplify the crucial host response to combat this disease. Cytokines are a class of soluble low molecular weight glycoproteins that possess a number of properties including mediating cell-to-cell communication during an immune response. Membrane-bound forms have also been described for many cytokines (81, 111). Cytokines are comprised of several groups of molecules including interleukins, lymphokines, monokines, interferons, colony-stimulating factors and a variety of other proteins (9, 81). Initially it was thought that these molecules were produced exclusively by leukocytes, lymphocytes, and monocytes; however, epithelial

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and endothelial cells secrete some as well (81, 216). Cytokines exhibit synergistic, antagonistic, redundant and pleiotropic properties (9, 216). Through these attributes this diverse group of molecules mediates, maintains, regulates and dictates the intensity and duration of the immune response in a coordinated, interactive way by modulating the activity of immune effector cells. The effects of cytokines on target cells are exerted by binding to specific membrane bound cytokine receptors which function as ligand binding and signal transduction moieties (81, 112, 111, 216, 239). The cellular consequences of ligand binding are diverse and involve the induction of gene transcription of cytokine encoding genes, as well as a diverse set of responses associated with cellular proliferation and differentiation (9). It has been suggested that the sharing of receptor subunits may in part reflect the redundant functions of cytokines (81, 111).

Functions of immune cells are finely coordinated to ensure an adequate response to an antigenic stimulus, and part of this coordination is mediated by secreted cytokines. Cytokines regulate the immune response by stimulating or alternatively inhibiting the proliferation of various cells or the secretion of antibodies or other cytokines. The development of an effective immune response involves lymphoid and inflammatory cells and other hematopoietic cells (134). Cytokines are secreted by various cells in the immune response such as T cells.

T cells can be subdivided based on their expression of CD4 or CD8

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membrane molecules. The $CD4^+$ T cells recognize antigen in association with major histocompatibility class II molecules (MHC) and largely function as helper cells, whereas $CD8^+$ T cells recognize antigen in association with MHC class I molecules and largely function as cytotoxic cells (241). $CD4^+$ helper T cells exist as two types in the mouse and are referred to as Th1 and Th2 cells (241). Th1 and Th2 cells show distinct patterns of cytokine production and effector function. Th1 cells secrete IL-2, TNF-ß, and IFN-γ and are the principal effectors of cell-mediated immunity against obligate or facultative intracellular pathogens and of delayed type hypersensitivity reactions (242, 243). Murine Th1 cells can also stimulate production of antibodies of the IgG2a class, which are effective at activating complement and opsonizing antibodies for phagocytosis. Th2 cells on the other hand produce IL-4 which stimulates IgE and IgG1 antibody production, IL-5, IL-10 and IL-13, which together with IL-4 downregulate macrophage functions (242). Th2 cell responses are associated with humoral immunity. Both classes of Th cell clones produce TNF-α, IL-3 and granulocyte macrophage colony stimulating factor (GM-CSF). In addition to Th1 and Th2 cells, $CD4^+$ Th cell subsets with a less differentiated cytokine profile than Th1 and Th2 cells, designated Th0 usually arise in the absence of clearly polarizing signals (325). It has been proposed that Th0 cells likely dominate the earliest stages of some responses and mediate intermediate effector functions, depending upon the spectrum

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and concentrations of cytokines produced and the nature of the responding cells. Th0 cells produce IL-2, IL-3, IL-4, IL-5, IFN-γ, TNF-α and GM-CSF (325). There is increasing data to support the view that these subpopulations also exist in humans (88, 298). Human Th1 and Th2 cells produce similar patterns as seen in the mouse, although synthesis of IL-2, IL-6, IL-10 and IL-13 is not as tightly restricted to a single subset as in murine T cells (243). The secreted cytokines of Th1 and Th2 cell types can mutually regulate and inhibit each other's functions. For example IFN-γ selectively inhibits proliferation of Th2 cells and IL-10 inhibits cytokine synthesis by Th1 cells (125, 242). It has been suggested that this cross regulation may partly explain the strong biases towards Th1 or Th2 responses during many infections in mice and humans. Therefore, the fine balance between the secreted cytokines is important for the resulting nature of host resistance against pathogens.

Th1 and Th2 patterns of cytokine secretion correspond to activated effector phenotypes generated during an immune response. They do not exist among naive T cells. When first stimulated by antigen on antigen presenting cells, naive $CD4^+$ T cells principally or entirely produce IL-2 and then differentiate into phenotypes that secrete other cytokines (328). Seder *et al.* (305) demonstrated that Th1 and Th2 cells can both be derived from a single precursor cell. IL-4 stimulates differentiation into Th2 cells, whereas IL-12 and IFN-γ enhance Th1 development (328). The factors that play a role in

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driving naive $CD4^+$ T cells toward Th1 or Th2-dominated populations include the type of antigen-presenting cells, the nature and amount of antigen, and other microenvironmental factors, such as hormones and cytokines (305, 189). In addition, the co-stimulatory proteins B7-1 and B7-2, located on the antigen-presenting cell, play important roles in initiating Th1/Th2 determination; however, their actions are more permissive than direct. For example, by initiating IL-4 production, B7-2 can have an effect that leads to Th2 cell development. Since B7-1 does not elicit IL-4 production, it leaves the cell open to other influences, such as IL-12 produced by macrophages. Kuchroo *et al.* (209) showed that anti-B7-1 added to an *in vitro* assay of mouse T cell differentiation led to increased production of IL-4 while anti-B7-2 led to greater IFN-γ production. Using human cells, Freeman *et al.* (133) also showed non-identical co-stimulatory signals from B7-1 and B7-2. Development of the appropriate Th subset during an immune response against offending agents is important because certain pathogens are more effectively controlled by either a predominantly Th1 or Th2 type immune response.

To date a detailed understanding of the host immune response to *L. pneumophila* has been hampered due to the lack of relevant systems to study infection. Several investigators have used *in vitro* systems to begin to elucidate the production of cytokines elicited by *L. pneumophila* or its antigens during infection of cells *in vitro*. Studies have demonstrated the

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importance of the macrophage-*L. pneumophila* interaction to allow *L. pneumophila* to persist in the lung (55, 57, 250). Furthermore, it is widely accepted that a cell-mediated host response is crucial to resolution of Legionnaires' disease (178, 369). Investigators have used a variety of *in vitro* models to study cellular infection by *L. pneumophila* and these include cells of human and non-human origin (224, 153, 187, 267, 368). Based on the nature of the disease process and the fact that *L. pneumophila* is a facultative intracellular pathogen, recent investigations have focused on understanding the interactions of *L. pneumophila* with the host's immune system.

L. pneumophila attachment to murine peritoneal macrophages has been shown to increase certain cytokine and chemokine mRNA levels (371). *Legionella* as well as purified antigens from this organism induce secretion of specific proinflammatory and immunoregulatory cytokines, such as IL-1β and IFN-γ respectively from a variety of cells including murine peritoneal, splenic and pulmonary macrophages as well as human peripheral blood monocytes and lymphocytes (203).

It is proposed that the resolution of *Legionella* infection is facilitated by activation of macrophages which subsequently resist *Legionella* infection. IFN-γ is a major macrophage activating factor and has been shown to activate human and rodent alveolar macrophages and monocytes to inhibit the growth of *L. pneumophila in vitro* but does exhibit bactericidal activity

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against *L. pneumophila* (65, 31, 149, 194, 249, 313, 314). In addition to IFN-γ, proinflammatory cytokines have been shown to play a role in infectious diseases. TNF-α participates in the cytokine cascade during the immune response, acting synergistically with IFN-γ. In addition, TNF-α has been shown to activate mononuclear phagocytes and rodent macrophages to inhibit intracellular parasites and to potentiate the antimicrobial action of IFN-γ (197, 314). *L. pneumophila* induces secretion of TNF-α *in vitro* and *L. pneumophila* infection leads to *in vivo* production of TNF-α, suggesting a potential role for this cytokine in host resistance to legionellosis (11, 37, 38). In contrast to activated monocytes and alveolar macrophages, PMNLs activated by IFN-γ and TNF-α have been shown to have an enhanced ability to kill *L. pneumophila* (38).

In addition to protective cytokines, such as IFN-γ and TNF-α immunosuppressive cytokines such as IL-10 are able to downregulate IFN-γ and TNF-α production which leads to exacerbated infection in many diseases (164, 173, 261, 293, 309). This effect has been seen with *M. leprae*, in which IL-10 is triggered in lepromatous patients with ineffective immune responses but not in tuberculoid leprosy patients that have an effective immune response (183). Thus, host resistance depends on the ability of the immune system to mount the appropriate cell response to the intracellular bacteria.

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Indeed, a cell-mediated immune response with a predominantly Th1 type cytokine pattern is crucial to recovery. Inhibitory cytokines have been shown to exhibit important deactivating effects on macrophages in murine models of infections due to *Leishmania* (22) *Trypanosoma* (310) *Toxoplasma* and *Schistosoma* (148) as well as, *Listeria* (343) and *Mycobacteria* (21, 20, 327) infections. Furthermore, IL-10 exacerbates *L. pneumophila* infection within resident alveolar macrophages and monocytes (264).

These data accumulated to date for cytokine production following *Legionella* infections has been obtained primarily using *in vitro* experimentation. The use of single cell systems provide limited insight into the complex interactions involved during infection of an animal host. Modulation of the immune system with cytokines requires a fine balance and specific interplay between cytokines and host cells in the immune response that cannot be accounted for by using single-cell systems. Animal models provide a means of testing theories of the pathogenesis of diseases such as *L. pneumophila* pneumonia as well as the potential value of therapeutic interventions.

Historically, guinea pig (132), rat (315) and hamster (263) models have been used to study the progression of Legionnaires' disease, but have not been useful for studying the host immune response to *L. pneumophila* infection, in part, due to a lack of specific immunologic reagents for these animal species. The use of animal models, primarily the guinea pig, have yielded

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insight into the underlying processes that occur following inhalation of *L. pneumophila* into the respiratory tract and have confirmed the host cellular responses to *L. pneumophila* challenge. Yamamoto *et al.* (370) established that peritoneal macrophages of A/J mice supported *L. pneumophila* replication, whereas peritoneal macrophages from other mouse strains including BALB/c, BDF1, C3H/HeN, C57BL/6 and DBA/2 were resistant to *Legionella* replication. Prior to these studies it was believed that mice were uniformly resistant to *L. pneumophila* infection and thus represented a poor model for studying Legionnaires' disease.

In recent years there has been an upsurge in interest to better understanding the host immune response, particularly the role that cytokines play, in regulating systemic infection. To date there is limited information regarding the production of cytokines during *L. pneumophila* infection in animal models. Brieland *et al.* (55) developed a surgical model of Legionnaires' disease pneumonia using the A/J mouse which has been shown in many ways to closely mimic the development of human disease. Furthermore, the abundance of immunologic reagents for the mouse has allowed subsequent study at the molecular level of the host's immune response in the lung following *L. pneumophila* infection.

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CHAPTER II

ADHERENCE OF *LEGIONELLA PNEUMOPIHLA* TO HOST CELLS AND THE ROLE OF THE 25 kDa MAJOR OUTER MEMBRANE PROTEIN OF *L. PNEUMOPHILA* IN ATTACHMENT TO U-937 CELLS.

(A portion of the work outlined in this section has been published as: Krinos, C., A. S. High and F. G. Rodgers. 1999. "Role of the 25 kDa major outer membrane protein of *Legionella pneumophila* in attachment to U-937 cells and its potential as a virulence factor for chick embryos". *J. Applied Microbiol.* 86:237-344.

2.1 Abstract

Adherence of *Legionella pneumophila* to alveolar macrophages is a necessary prelude to infection in the lung. The attachment of the organism to U-937 cells, A549 cells and primary explants of thioglycollate-elicited peritoneal macrophages from A/J mice was investigated in a opsonin-independent system. Bacterial attachment to host cells was determined by viable bacterial cell colony (VBCC) counts and indirect immunofluorescence assay (IFA) methods. *L. pneumophila* attached to the three cell types investigated in the absence of opsonins and maximum binding occurred at a multiplicity of infection (MOI) of 100. Following these initial adherence

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studies, U-937 cells were used further to investigate the role of the 25 kDa major outer membrane protein (MOMP) present on the surface of *L. pneumophila* as an adhesin for the organism. The gene encoding the 25 kDa MOMP of *L. pneumophila* was previously transformed into *Escherichia coli* JM 83 and the resultant *E. coli* LP 116 clone was shown to express the *Legionella*-MOMP. Compared with the parent *E. coli* JM 83 strain, the *E. coli-Legionella*-MOMP-containing clone showed a 5-fold increase in opsonin-independent binding to U-937 cells as assayed by both VBCC counts and IFA techniques. In addition, the clone showed enhanced virulence for chick embryos. These studies suggest that the 25 kDa MOMP of *L. pneumophila* functions as an adhesive molecule for host cells and serves as a virulence factor for the organism.

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2.2 Introduction

Legionella pneumophila is a gram-negative, facultative intracellular pathogen responsible for a necrotizing, lobar pneumonia known as Legionnaires' disease. The bacterium is capable of replicating in a variety of cell types, yet primarily parasitizes alveolar macrophages during human disease (250).

Attachment of bacteria to host cells is usually quite specific and is determined by the presence of specific ligand or adhesin molecules on the surface of the microorganism that recognize receptors on host cells (24). As such, binding is regarded as a prerequisite for tissue invasion and infection and this initial interaction has been the subject of numerous reports including those on *Neisseria* spp., *Escherichia coli, Vibrio cholerae, Pseudomonas aeruginosa* and *Streptococcus* spp. (91, 123, 257, 270, 366). In these investigations some of the factors responsible for disease initiation have been defined. Many pathogens possess pili, fimbriae, capsular mucopolysaccharide, flagella, lectin-like molecules, and OMPs all of which have been implicated in bacterial attachment to host cells (91, 123, 287, 317). Several factors contribute to the attachment process, including availability of appropriate receptors, attractive and repulsive forces (van der Waals forces), pH and the hydrophobicity of the interacting membranes (24, 104). Binding can be divided into a two step process in which a preliminary or weak binding event is followed by a firm highly specific attachment process. Several

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pathogenic bacteria use a group of highly conserved immune system-specific receptors known as integrins, specifically those of the β1 and β2 subsets, to gain entry into various cells (8, 26, 82, 192, 274, 303). The β1 integrins are used by organisms that either possess invasin on their surface while the β2 group, which includes complement receptor type 3 (CR3) are used by organisms that directly bind to these cells in the presence or absence of complement or immune modulators (8, 26, 93, 155, 192, 286, 303).

Two mechanisms of attachment have been proposed for *L. pneumophila;* opsonin-dependent (26, 266) and opsonin-independent systems (154, 286). Opsonins are molecules that enhance phagocytosis and include antibody and complement which facilitate antigen clearance. Thus, in a complement-mediated system, antibody and complement bind to bacterial surfaces increasing the engulfment of the antigen by macrophages. In the complement-mediated system, complement receptors CR1 and CR3 on human professional phagocytes that recognize complement fragments C3b and C3bi respectively mediate phagocytosis of *L. pneumophila* (26, 266). The mechanism of direct recognition of bacteria by macrophages in the absence of opsonic components is not well defined. However, given the low opsonic components present in the lung, an opsonin-independent mechanism of attachment may be critical for disease establishment (277). It has been hypothesized that organisms which enter host cells via this system are able to

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survive the antimicrobial processes induced in the cell by the opsonin-mediated event and that these continue to grow and cause severe disease. This non-opsonic, direct binding type of recognition has been established for many pathogens, such as *M. tuberculosis* , group B streptococci and *Yersinia pseudotuberculosis* and may represent a crucial mechanism for disease establishment (8, 82, 191). Indeed, there is evidence that the intracellular fate of pathogens is dictated by the initial mode of attachment (82, 346, 359, 366).

The outer membrane content of *Legionella* has been explored to determine if a single factor is responsible for uptake and intracellular survival of the organism and several genes have been identified that are responsible for intracellular survival, evasion of endocytic pathways and killing of host cells (48, 74, 271, 350). Although reports have varied as to the commonality of various proteins across strains of *L. pneumophila* and *Legionella* species, some *L. pneumophila* serogroups do contain a single MOMP that ranges in mass between 24 to 29 kDa (63, 170, 172). Butler and Hoffman (62) have also reported the presence of 28 and 31 kDa outer membrane proteins (OMP) in *L. pneumophila*.

In the complement-mediated system, the MOMP of *L. pneumophila* has been shown to selectively fix the complement component C3 and this facilitates uptake by complement-mediated phagocytosis (26). Gibson *et al.* (154) showed that treatment of host cells with monoclonal antibodies (MAb)

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specific for CR1 (CD35), CR3 (CD11b + CD18) and CR4 (CD11c + CD18) to block these receptors or their sub-units did not interrupt bacterial attachment or intracellular replication. The target cell for *L. pneumophila* infection during human disease is the alveolar macrophage, thus, this cell is ideal for studying *Legionella*-host interactions; however, obtaining these cells is extremely difficult. A wide variety of eukaryotic cells have been shown to support *L. pneumophila* replication *in vitro* (188, 202, 224, 250, 286) Pearlman *et al.* (267) demonstrated that the transformed macrophage-like U-937 cell, established from a human histiocytic lymphoma, supported the intracellular survival of *L. pneumophila*. *L. pneumophila* possess pili, fimbriae and flagella and as suggested by Rodgers (287), these may potentiate binding by overcoming the gap between the negatively charged surfaces of the invading pathogen and the host cells. In addition, Oldham and Rodgers (258) have investigated *L. pneumophila* interaction with HEp-2 cells, Vero cells and MRC-5 cells, the later being of human embryonic lung origin. It was demonstrated that *L. pneumophila* attachment to cells resulted in thickening of eukaryotic cell membranes and proliferation of microvilli leading to bacterial endocytosis, referred to as "bacteriopexis".

In the present study, the first objective was to investigate opsonin-independent adherence of *L. pneumophila* using two human transformed cell lines (U-937 and A549) and a primary murine cell (A/J peritoneal macrophages) as a potentiator for infection. U-937 cells are transformed

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macrophage-like cells, established from a human histiocytic lymphoma (326) and A549 cells are human transformed cells derived from explant cultures of lung carcinomatous tissue (151) and have been characterized as type II pneumocytes (215). Following these experiments the role of the MOMP of *L. pneumophila* as a direct adhesin to U-937 cells was investigated using a recombinant clone which expresses the MOMP. Adherence assays were evaluated by both VBCC counts and IFA assays and the ability and number of bound organisms following adherence was determined. Studies to elucidate the bacterial adhesin of *L. pneumophila* may serve to assist in development of novel strategies for the prevention and control of Legionnaires' disease, especially in high risk groups. Anti-adherence vaccines and purified adherence molecules used in a competitive fashion were evaluated by Beachey (24) and yielded promising results.

2.3 Materials and Methods

Reagent formulations and preparation along with detailed procedures are given in Appendix 1.

2.3.1 Organism Cultivation:

Legionella pneumophila. *L. pneumophila* serogroup 1, strain Nottingham 7 is a highly virulent clinical isolate from a fatal case of Legionnaires' disease. The organism was initially isolated from sputum on

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low sodium, enriched blood agar. Colonies were sub-cultured once on buffered charcoal yeast extract agar supplemented with L-cysteine, ferric pyrophosphate and α-ketoglutarate (BCYE-α) and stored as stock cultures frozen at -70°C in 1 % serum sorbitol (Appendix 1). Thawed aliquots were inoculated onto BCYE-α and incubated for 72 h at 37°C. Colonies were harvested, placed into buffered yeast extract broth supplemented with L-cysteine, ferric pyrophosphate and α-ketoglutarate (BYE-α) at a density of $1x10^6$ CFU (colony forming units)/ml and bacteria were grown for 24 h at 37°C with shaking, yielding approximately $1x10^{10}$ CFU/ml as determined by VBCC counts. *L. pneumophila* were harvested from broth by centrifugation at 6,000 x g in a Beckman Microfuge 12 (Beckman Instruments, Palo Alto, CA.), washed three times with Hanks Balanced Salt Solution (HBSS) (Cellgro, Herndon, VA.) and adjusted to $5x10^9$ CFU/ml. Serial 10-fold dilutions were made from $5x10^9$ to $5x10^5$ in order to yield the various bacteria to host cell ratios. For subsequent adherence assays bacteria were adjusted to $5x10^7$ CFU/ml. The ratio of bacteria to cells was approximately 100:1 for all cell lines used in these experiments. A MOI of 100 was chosen because bacterial binding experiments using U-937 cells confirmed previously established data conducted in our laboratory that an MOI of 100 reached near maximal binding capacity to host cells. In addition, bacterial binding experiments were performed using A549 and murine peritoneal macrophages with similar

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results.

***Escherichia coli* JM 83 and *E. coli* JM 83 LP clone 116.** *E. coli* JM 83 (JM 83) is a laboratory strain with the ability to express cloned fragments and supports the multiplication of extra-chromosomal elements. *E. coli* JM 83 LP clone 116 (LP 116) is a genetically mutated derivative of *E. coli* JM 83 generated in our laboratory by High *et al.* (170) using pUC 19 as a vector. This strain has been shown to express the MOMP of *L. pneumophila* and is resistant to ampicillin. The clone was grown on nutrient agar or in nutrient broth containing 50 μg/ml ampicillin (Appendix 1). All strains were stored frozen at -70°C in serum sorbitol. JM 83 or LP 116 were thawed and inoculated onto nutrient agar and allowed to grow for 24 h at 37°C. A 100 μl aliquot of nutrient broth suspension of JM 83 (62 Klett units) or LP 116 (60 Klett units) was added to 5 ml sterile nutrient broth ($5x10^5$ CFU/ml). These suspensions were incubated in a shaking incubator for 6 h (JM 83) or 8 h (LP 116) at 37°C and adjusted to give approximately $5x10^7$ CFU/ml prior to adherence assays.

2.3.2 Eukaryotic Cell Growth and Maintenance:

U937 cells. U-937 cells (ATCC, Rockville, MD) express macrophage-like characteristics when treated with phorbol esters (267). The cells were maintained as replicative, non-adherent monocyte-like cells grown in RPMI-1640 (Cellgro) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO.) and 3 mM L-glutamine (Sigma)

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(Appendix 1). For adherence assays, cells were grown to 1-2x10^6 cells/ml in T-75 cm^2 flasks (Costar, Cambridge, MA.) and harvested by centrifugation at 250 x g for 10 min at 4°C. Cells were then resuspended in 1 ml fresh medium and counted using trypan blue vital stain (0.04 %). Cells were then adjusted to 1x10^7 cells/ml in fresh medium, treated with phorbol 12-myristate 13-acetate (PMA) (Sigma) (final concentration of 10^{-8} M) and cultured for 24 h at 37°C with 5 % CO_2 in T-75 cm^2 flasks. Adherent cells were washed three times with HBSS, physically removed from the culture flasks by gentle scraping with a silicon rubber-coated glass rod and resuspended in fresh medium to 5x10^5 cells/ml for VBCC counts. A 1 ml aliquot of this suspension was placed into each well of 24-well cell culture plates (Costar) and allowed to re-adhere for 24 h. For IFA assays, U-937 cells were adjusted to 1x10^6 cell/ml and 2 ml of the suspension was placed into 6-well plates containing 484 square mm glass coverslips and were allowed to readhere for 24 h at 37°C with 5 % CO_2.

A549 cells. A549 cells, an epithelial, adherent cell line characterized as human type II pneumocytes, were obtained from ATCC and maintained in HAM's FK12 medium supplemented with 10 % FBS and 3 mM L-glutamine (Appendix 1) in T-75 cm^2 flasks. For bacterial attachment assays, adherent cells were washed three times with HBSS without Ca^{++} or Mg^{++} and treated with trypsin-EDTA (Cellgro) solution for 1 minute. Fresh medium was added

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and cells were collected and centrifuged at 250 x g for 10 min at 4°C, resuspended in 1 ml fresh medium and counted using trypan blue vital stain. Cells were distributed into 24-well cell culture plates at $5x10^5$ cells/ml and allowed to re-adhere for 2 h at 37°C with 5 % CO_2. For IFA assays, 2 ml of a $1x10^6$ cell/ml suspension was placed into 6-well cell culture plates containing 484 square mm glass coverslips and cells were allowed to re-adhere for 2 h.

A/J mice peritoneal macrophages. A colony of A/J mice was established at the University of New Hampshire animal facility by breeding siblings from the same liter (Jackson Laboratories, Bar Harbor, Maine). Mice received food and water ad libitum and were housed, cared for and bread in accordance with NIH and University of New Hampshire animal care and use committee guidelines under ACUC protocol # 960905. Mice 6-8 weeks old were injected intraperitoneally with 2 ml of a 3 % Brewer's thioglycollate solution 3 days prior to harvesting macrophages. Mice were humanely euthanized by CO_2 asphyxiation, and peritoneal macrophages were collected by injecting 10 ml of HBSS without Ca^{++} and Mg^{++} into the peritoneal cavity using a 26-gauge needle. Fluid was retrieved from the peritoneal cavity using an 18-gauge needle and all fluids were pooled into a 50 ml conical tube and centrifuged at 250 x g for 10 min at 4°C. Cells were washed three times with HBSS, resuspended in RPMI-1640 supplemented with 10 % heat-inactivated FBS and 3 mM L-glutamine. Cell suspensions were counted and cells were

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distributed into either 24-well or 6-well cell culture plates at $5x10^5$ cells/ml or $1x10^6$ cell/ml respectively and allowed to re-adhere for 2 h at 37°C with 5 % CO_2.

2.3.3 Adherence Assays:

L. pneumophila was grown in BYE-α broth for 24 h as described in section 2.3.1. Following washing steps with HBSS, for eukaryotic cells and bacteria, bacteria were diluted in serum-free HBSS corresponding to MOI's of 1, 10, 100, 1000 or 10,000 to determine the maximum bacterial binding capacity for all cell types. In addition, to evaluate the role of the MOMP as an adhesin for *L. pneumophila,* bacterial cultures (*L. pneumophila* , *E. coli* JM 83 and clone LP 116) were centrifuged at 6,000 x g, washed three times with HBSS and resuspended in 1 ml fresh HBSS. Each cell type was treated with cytochalasin D at 1 μg/ml in HBSS for 1 h at 37°C with 5 % CO_2 to inhibit bacterial uptake. Cells were washed three times with HBSS to remove cytochalasin and each MOI was added to 24-well or 6-well cell culture plates containing either U-937, A549 or thioglycollate-elicited A/J peritoneal macrophages for 1 h. For MOMP studies, organisms were added to U-937 cells at a MOI of 100 bacteria per host cell and incubated for 1 h at 37°C in 5 % CO_2. Cells were washed three times with HBSS to remove unbound bacteria and assayed by VBCC counts or IFA.

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2.3.4 Viable bacterial cell colony (VBCC) counts. Eukaryotic cells cultured with viable *L. pneumophila* were subsequently assayed for numbers of bound bacteria by adding 1 ml of sterile water to each well followed by incubation at room temperature for 20 min. Cells were disrupted with vigorous aspiration and expulsion through a sterile pasteur pipet. A 100 µl aliquot of each suspension was serially 10-fold diluted in 1 % (w/v) peptone and duplicate 25 µl samples of each dilution were plated onto BCYE-α. *Legionella* colonies were enumerated after 72 h and the number of bacteria bound to each cell type was determined by dividing the average lysate values for each well by the number of eukaryotic cells seeded per well. Three separate assays were performed with each MOI in triplicate. Following evaluation of binding data, adherence assays were repeated three times using an MOI of 100 for all cell types. For MOMP studies, duplicate 25 µl samples of each dilution were plated on either BCYE-α (*L. pneumophila*), nutrient agar (JM 83) or nutrient agar supplemented with 50 µg/ml ampicillin (LP 116). Organisms were enumerated by counting bacterial colonies.

The number of virulent *L. pneumophila* organisms binding to the U-937 cells was defined as the 100 % adherence value and the binding potential of all other strains to host cells was compared to this. In this fashion, variations in attachment capabilities between strains were determined and increases or decreases greater than 50 % were considered significant. Results were expressed as the average of three separate trials each conducted in

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triplicate.

2.3.5 **Immunofluorescence (IFA) assay.** Prior to addition of eukaryotic cells to the 6-well cell culture plates, 484 square mm sterile glass coverslips were added to each well and the cells were allowed to adhere to the coverslips. Monolayers were cytochalasin D treated, washed and inoculated with *L. pneumophila*, *E. coli* JM 83 or *E. coli* LP 116 as described for VBCC counts. Cells were washed three times with PBS and treated with a 1:500 dilution of rabbit polyclonal anti-*L. pneumophila* N7 or anti-*E. coli* JM 83 serum (Appendix 1) for 1 h at 37°C. After 1 h of incubation, host cells were washed three times to remove non-adherent bacteria and fixed in 10 % formalin in phosphate-buffered saline, pH 7.2 (PBS) for 1 h at 25°C. Cells were washed three times to remove unbound globulin and treated with a 1:500 dilution of goat anti-rabbit fluorescein isothiocyanate-conjugated (FITC) antibody for 1 h at 37°C. Cells were washed with PBS and stained with 0.01 % propidium iodide (Sigma) in PBS for 20 min. Glass coverslips were mounted on glass slides in glycerin containing 1 % (w/v) 1,4-diazobicyclo (2.2.2.) octane (DABCO) (Sigma) and viewed with an Olympus BH-2 epifluorescence microscope using an excitation filter emitting a wavelength of 490 nm and a barrier filter blocking wavelengths above 515 nm. IFA results were averages of three separate trials each conducted in duplicate enumerating the number of adherent bacteria on the first 200 cells per trial counted and expressed as adherent organisms/host cell.

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2.4 Results

Adherence of *L. pneumophila* to U-937, A549 and A/J peritoneal macrophages. *L. pneumophila* attachment to U-937 cells, A549 cells and A/J murine peritoneal macrophages was investigated at various MOI's by both VBCC counts and IFA methods. Bacterial binding to U-937, A549 and A/J peritoneal macrophages is shown by IFA in Plate 2.1. Maximum bacterial binding occurred at an MOI of 1000 ($5x10^8$ CFU/ml) for U-937 cells (Figure 2.1 and 2.2), A549 cells (Figure 2.3 and 2.4) and A/J peritoneal macrophages (Figure 2.5 and 2.6) as assayed by both VBCC counts and IFA. Maximum binding corresponded to an average CFU/cell ratio of 1.97, 3.04, and 1.15 for U-937 (Figure 2.1), A549 (Figure 2.3) and A/J peritoneal macrophages (Figure 2.5) respectively as assayed by VBCC counts. IFA equivalent ratios were 2.92, 2.7, and 1.4 for U-937 (Figure 2.2), A549 (Figure 2.4) and A/J peritoneal macrophages (Figure 2.6). There was a high level of non-specific binding to the plastic culture ware and glass coverslips with an MOI of 1000 ($5x10^8$ CFU/ml), however, when 10-fold fewer organisms were used, corresponding to an MOI of 100 ($5x10^7$ CFU/ml), the level of non-specific binding was reduced to acceptable levels. Bacterial binding at an MOI of 100 approached maximum binding levels for all cell types and was subsequently used for all additional assays.

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Plate 2.1: *L. pneumophila* binding to eukaryotic cells in an indirect immunofluorescence assay. Bacteria were labelled with FITC (green) while eukaryotic cells were counterstained with propidium iodide (orange). A) *L. pneumophila* binding to U-937 cells. B) *L. pneumophila* binding to A549 cells. C) *L. pneumophila* binding to thioglycollate-elicited peritoneal macrophages from A/J mice.

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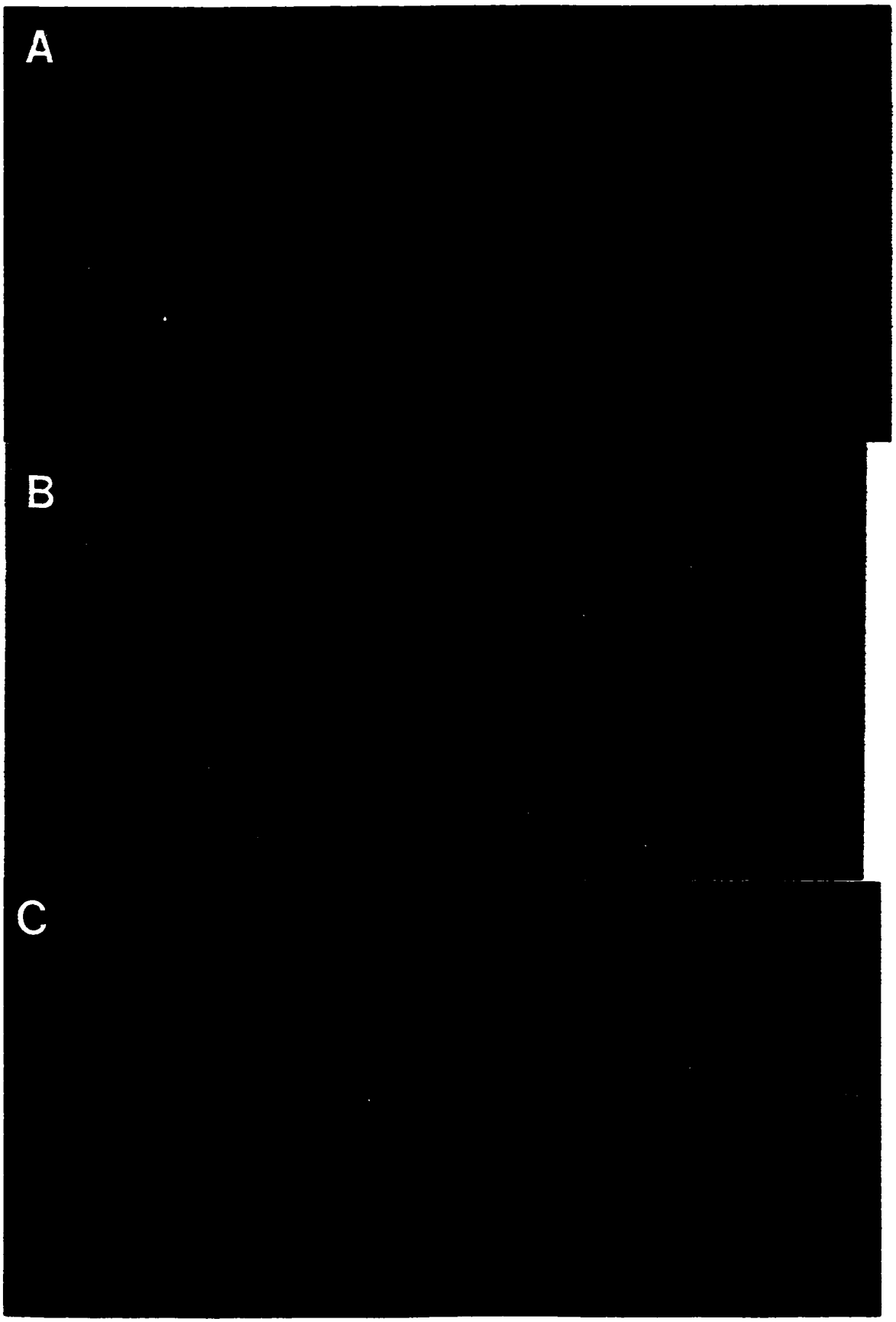

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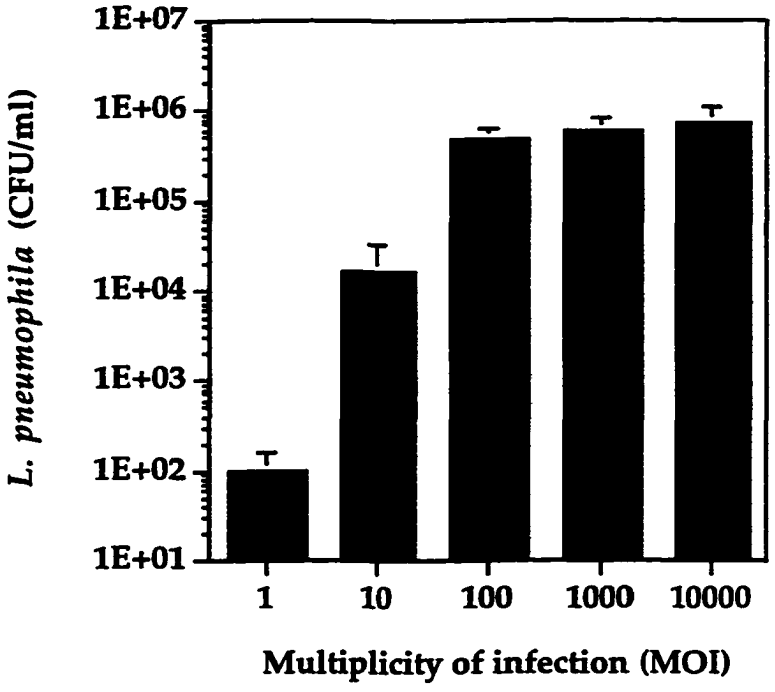

Figure 2.1: Binding of *L. pneumophila* to U-937 cells as measured by VBCC counts. U-937 cells were seeded at $5x10^5$ cells/well and increasing 10-fold multiplicities of infection of bacteria were added to the cells. An inoculum of $5x10^7$ CFU/ml approached maximum binding of *L. pneumophila* corresponding to 1.12 bacteria per U-937 cell. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate (i.e. 9 data points per column).

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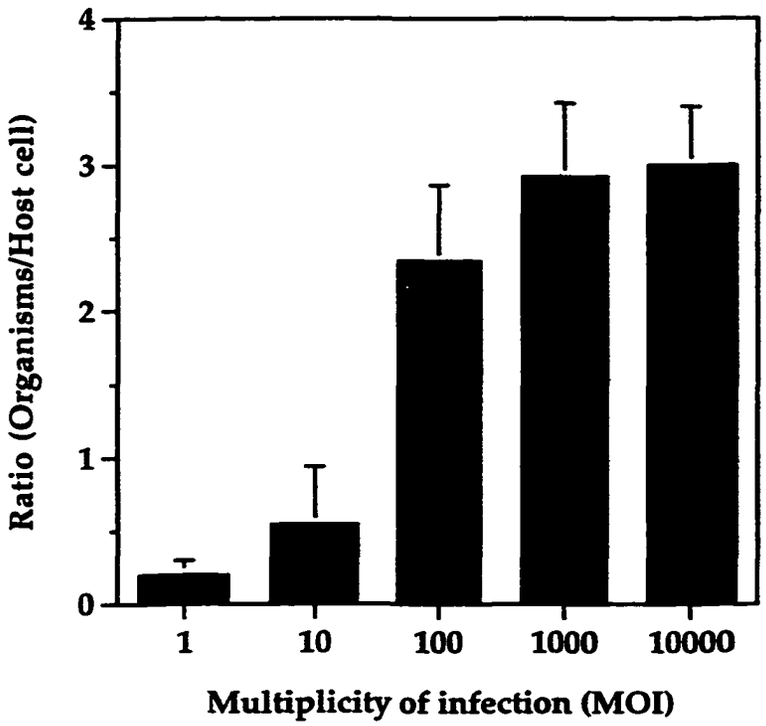

Figure 2.2: Binding of *L. pneumophila* to U-937 cells as measured by IFA. U-937 cells were seeded at $2x10^6$ cells/well and increasing 10-fold multiplicities of infection of bacteria were added to the cells. An inoculum of $2x10^8$ CFU/ml approached maximum binding of *L. pneumophila* corresponding to 2.34 bacteria per U-937 cell. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate enumerating the total number of fluorescent bacteria bound to 200 host cells (i.e. 9 data points per column).

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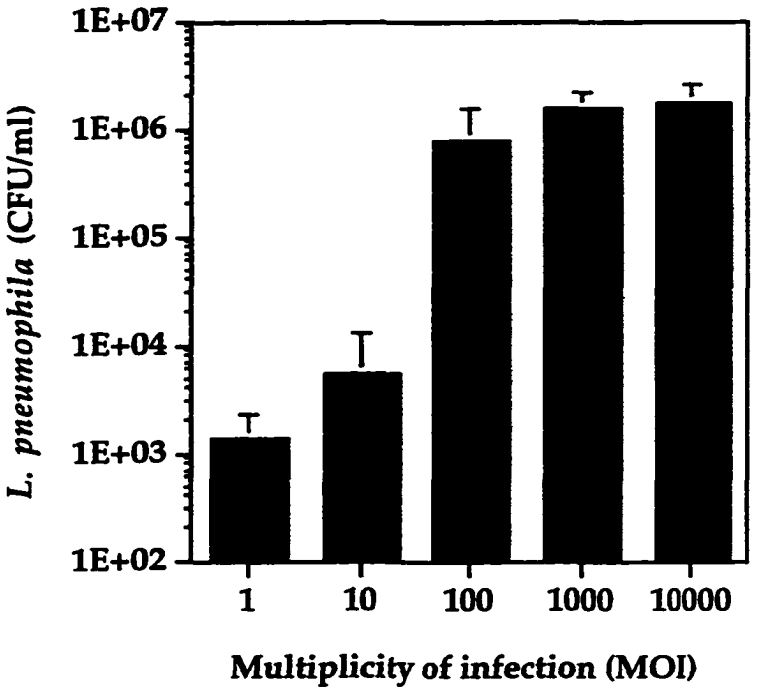

Figure 2.3: Binding of *L. pneumophila* to A549 cells as measured by VBCC counts. A549 cells were seeded at $5x10^5$ cells/well and increasing 10-fold multiplicities of infection were added to the cells. An inoculum of $5x10^7$ CFU/ml approached maximum binding of *L. pneumophila* corresponding to 1.6 bacteria per A549 cell. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate (i.e. 9 data points per column).

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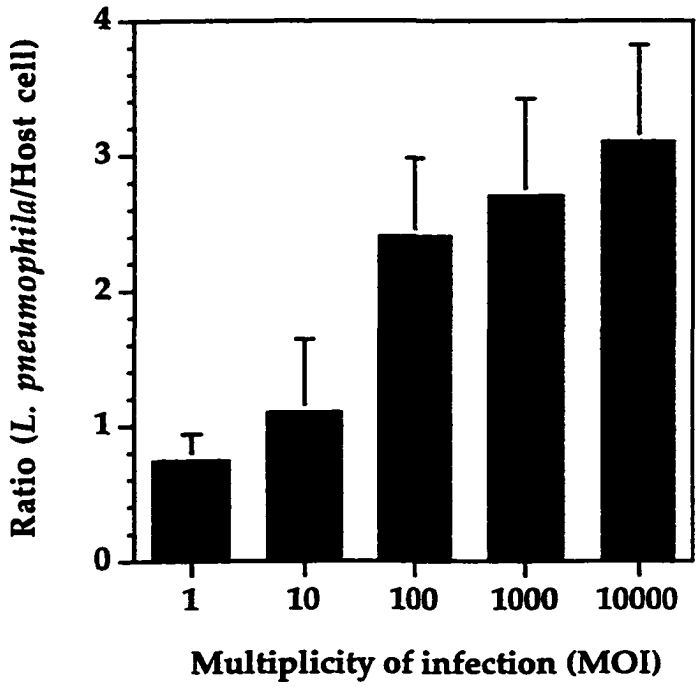

Figure 2.4: Binding of *L. pneumophila* to A549 cells as measured by IFA. A549 cells were seeded at $2x10^6$ cells/well and increasing 10-fold multiplicities of infection of bacteria were added to the cells. An inoculum of $2x10^8$ CFU/ml approached maximum binding of *L. pneumophila* corresponding to 2.3 bacteria per A549 cell. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate enumerating the total number of fluorescent bacteria bound to 200 host cells (i.e. 9 data points per column).

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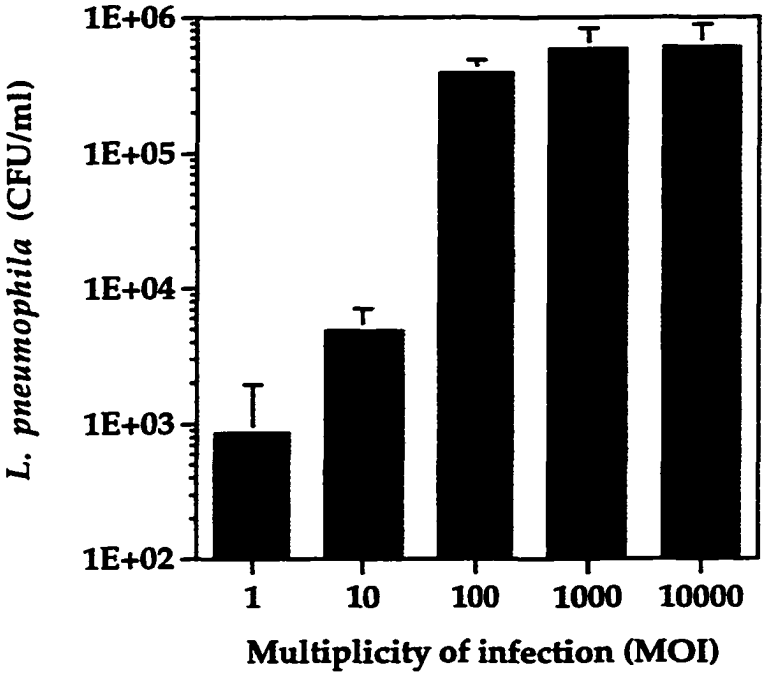

Figure 2.5: Binding of *L. pneumophila* to thioglycollate-elicited peritoneal macrophages from A/J mice cells as measured by VBCC counts. Macrophages were seeded at $5x10^5$ cells/well and increasing 10-fold multiplicities of infection were added to the cells. An inoculum of $5x10^7$ CFU/ml approached maximum binding of *L. pneumophila* corresponding to 0.77 bacteria per A/J macrophage. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate (i.e. 9 data points per column).

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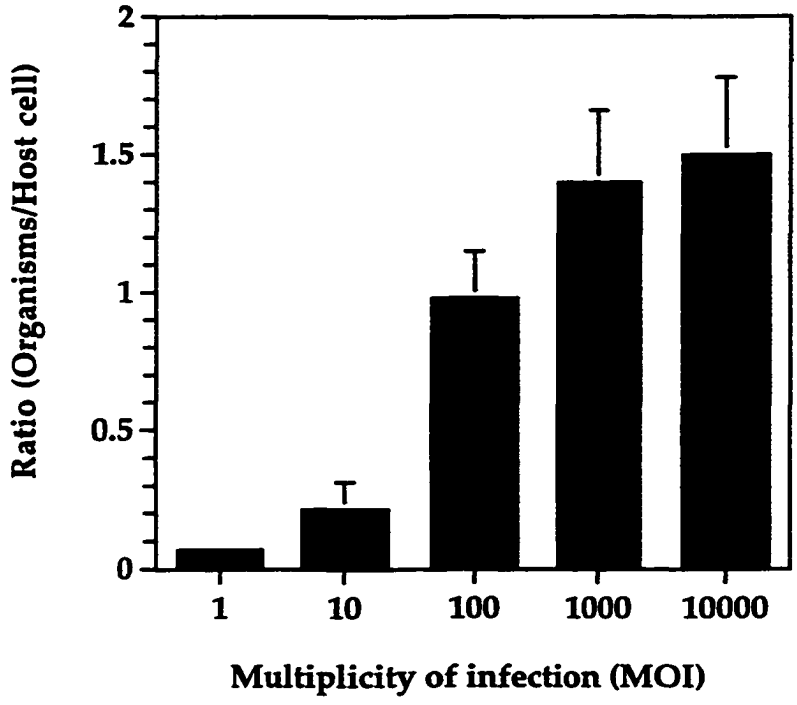

Figure 2.6: Binding of *L. pneumophila* to thioglycollate-elicited peritoneal macrophages from A/J mice as measured by IFA. Macrophages were seeded at $2x10^6$ cells/well and increasing 10-fold multiplicities of infection of bacteria were added to the cells. An inoculum of $2x10^8$ CFU/ml approached maximum binding of *L. pneumophila* corresponding to 0.98 bacteria per macrophage. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate enumerating the total number of fluorescent bacteria bound to 200 host cells (i.e. 9 data points per column).

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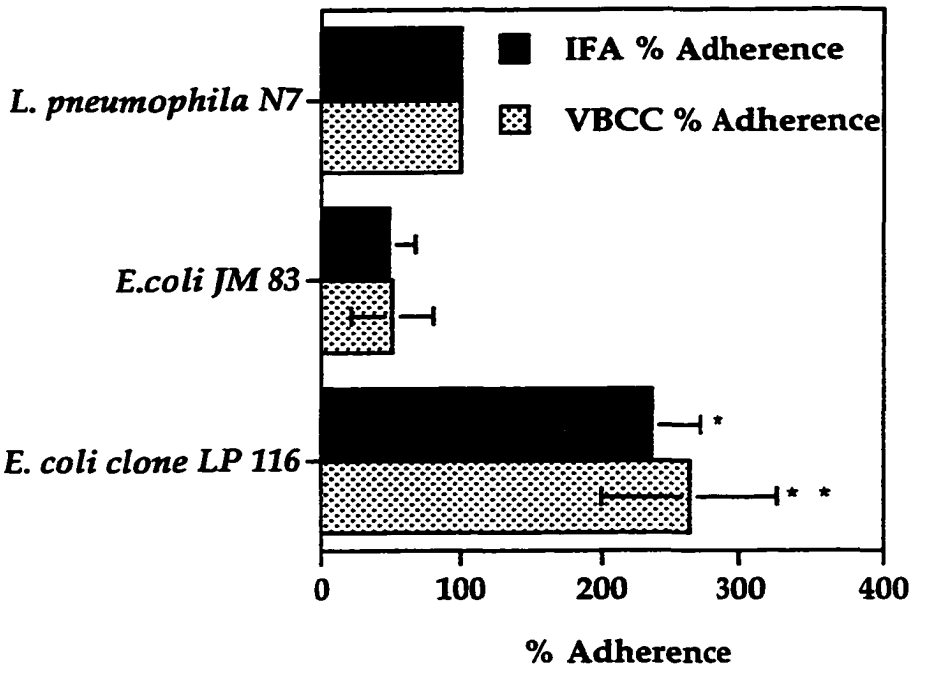

Figure 2.7: Adherence of *L. pneumophila* strain N7, *Escherichia coli* strain JM 83 and the *E. coli* clone LP 116 to U-937 cells assayed by IFA and VBCC counts. Data have been normalized to *L. pneumophila* which received a 100 % adherence value. *E. coli* clone LP 116 was 4.8 and 5.1 times more adherent than the parent *E. coli* JM 83 strain as assayed by IFA or VBCC counts respectively. Each bar represents the average of three trials. *$p < 0.0004$, **$p < 0.0001$.

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The role of the MOMP as an adhesin in the binding of *L. pneumophila* to macrophage-like U-937 cells in an opsonin-independent environment was assessed. This was accomplished by comparing the attachment of the parent *E. coli* JM 83, and the genetically mutated *E. coli* JM 83 clone LP 116 which expresses the MOMP gene of virulent *L. pneumophila*. Comparison of *E. coli* JM 83 and LP 116 showed that the presence of the MOMP gene facilitated organism binding in the absence of opsonins. LP 116 was 5 times more adherent than the parent strain by both VBCC ($p < 0.0001$) counts and IFA ($p < 0.0004$) (Figure 2.7).

2.5 Discussion

Attachment of bacteria to its host cell is a necessary event for the disease process to occur (24). Several adherence mechanisms and bacterial components, including pili, fimbriae, flagella and outer membrane proteins have been shown to mediate the specific binding event (24, 47, 323, 269). However, other processes may be involved in the initial stages of bacteria-host cell interaction, such as electrostatic, van der Waals and hydrophobic interactions which are followed by stronger binding events. Several transformed cells have been proposed as potential cell models for *L. pneumophila* infection (155, 224, 258, 267). To evaluate attachment of *L. pneumophila* to eukaryotic cells in an opsonin-independent environment, three cell types were used in the adherence assays, the U-937 cell, the A549 cell

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and A/J thioglycollate-elicited peritoneal macrophages. Each cell was chosen to serve a particular purpose. The U-937 cell is a human histiocytic lymphoma cell line which expresses either monocyte or macrophage-like cell surface markers (326) and has been used frequently as a model to represent *Legionella*-host cell interactions (155, 154, 267, 286). U-937 cells are maintained as non-adherent monocyte-like cells, however, they can be differentiated into macrophage-like cells when stimulated with various agents, such as phorbol myristate acetate (PMA), interferon-γ (IFN-γ), 1-α-dihdroxyvitamin D3, human recombinant interleukin-6 or retinoic acid. When differentiated, these cells possess receptors analogous to macrophages, adhere to culture vessels, and are incapable of cell replication. These cells express both high and low IgG binding receptors as well as complement receptor type 3 (CR3) and complement receptor type 4 (CR4) at levels similar to alveolar macrophages. Thus, this cell is considered a versatile and appropriate cell model for many pathogens, including *L. pneumophila* and thus it was chosen to further investigate the role of the 25 kDa MOMP of *L. pneumophila*.

The A549 cell is a human, epithelial, transformed cell characterized as a type II pneumocyte (151, 215). Type II pneumocytes line the alveolus in the lung and may serve to perpetuate disease by supporting *L. pneumophila* binding and subsequent intracellular replication. In addition *L. pneumophila* may interact with these cells stimulating them to produce immune

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modulating molecules, such as cytokines that may either exacerbate or suppress disease.

Transformed cells are of crucial importance for studying the pathogenesis, microbiology and immunology of bacteria-host interactions. However, these cells do not directly mimic cells that are found *in vivo* in an unaltered state. Therefore, peritoneal cells from the susceptible A/J mouse strain, were used to represent a macrophage cell found in its natural state *in vivo*. Yamamoto *et al.* (370) demonstrated that thioglycollate-elicited peritoneal macrophages from various mouse strains were not susceptible to *L. pneumophila* infection with the exception of the A/J strain.

During the adherence assays, cytochalasin D was used to inhibit the internalization of *L. pneumophila*. The cytochalasins have been used to determine if pathogens enter host cells by means of active invasion or phagocytosis (105). It has been demonstrated that *L. pneumophila* do not actively penetrate cytochalasin-treated guinea pig alveolar macrophages *in vitro* without affecting attachment to host cell membranes (105). In addition to adhering to phagocytic-type cells, such as U-937 cells and A/J peritoneal macrophages, *L. pneumophila* were able to adhere to A549 cells, indicating that this organism adheres to cells that do not possess complement and Fc receptors normally found on the surface of mononuclear cells; thus, an alternative mechanism may be responsible for bacterial binding to these cells.

As a facultative intracellular pathogen, *L. pneumophila* interacts with

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the cell membranes of alveolar macrophages prior to phagocytosis and, as a result, surface-located antigens are crucial to disease induction. The MOMP of *L. pneumophila* is known to be tightly associated with LPS and resists complete dissociation in SDS at 100°C (141, 172). As a consequence of difficulties associated with separating the MOMP completely from LPS, recombinant DNA techniques were used to obtain *Legionella* surface expressed antigens. High *et al.* (170) constructed a recombinant plasmid containing an 810 base pair DNA fragment from the low-passage, fully virulent *L. pneumophila* N7 strain. The plasmid, pLP 116, was shown to express the 25 kDa *L. pneumophila* MOMP in *E. coli* by both Western blot and immunofluorescence analysis using an anti-*Legionella* MOMP-specific MAb. The genetically altered *E. coli* clone, LP 116, was used to assess the role of this 25 kDa protein in the pathogen-host cell adherence phenomenon. It was demonstrated that the presence of the 25 kDa MOMP enhanced the ability of the organism to attach to host cells. The 5-fold increase in binding to U-937 cells observed for the clone as compared to the parent *E. coli* suggested that the MOMP was, in part, responsible for this opsonin-independent attachment. It is possible that the high expression pUC 19 vector used in these studies resulted in an increase in the amount of MOMP on the surface of LP 116 organisms and that this expression was responsible for the greater than two-fold increased binding of the clone to U-937 cells as compared to the virulent *L. pneumophila* N7 strain. Alternatively, the attachment potential

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of the clone may have been influenced by conformational changes in the expression of the MOMP in the *E. coli* clone. Furthermore, it is possible that the MOMP of *L. pneumophila* is not associated with LPS in the clone; thus, steric factors may have contributed to the observed increase in adherence. Further studies on these strains will yield information on the nature of the MOMP adhesin-host cell receptor interaction. Given the role of the 25 kDa MOMP in virulence and in binding the organism to host cells, it is possible that this protein may serve as a useful moiety for the development of molecular vaccines against Legionnaires' disease.

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CHAPTER III

CELLULAR MODELS OF INFECTION FOLLOWING OPSONIN-INDEPENDENT ADHERENCE OF *LEGIONELLA PNEUMOPHILA*

3.1 Abstract

Elucidating the specific interactions of *Legionella pneumophila* during infection requires the use of cell models in order to develop a thorough understanding of the bacteria-host cell relationship. A cell model of infection of U-937 cells, A549 cells and thioglycollate-elicited peritoneal macrophages from A/J mice by *L. pneumophila* was assessed and established. Previous investigations have shown that several cells support the replication of *L. pneumophila in vitro*. There is a need to delineate the interactions of *L. pneumophila* with its primary host cell during human infection. The alveolar macrophage, the target cell in Legionnaires' disease, is, however, difficult to obtain. Therefore, an alternative cell model that mimics *L. pneumophila* infection of alveolar macrophages is required. In addition, the interaction between *L. pneumophila* and other cell types, such as the epithelial pneumocyte-like A549 cell will help to define *L. pneumophila*'s ability to sustain infection by replicating within cells other than alveolar

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macrophages thus prolonging its existence and possibly aiding in pathogenesis. Growth kinetics of *L. pneumophila* revealed that bacteria were able to attach to, penetrate and replicate within U-937 cells, A549 cells and A/J peritoneal macrophages in an opsonin-independent fashion. Bacteria bound to host cells within 1 h and replicated over a 36 h period at which time organisms were released from infected cells by lysis. This work demonstrates that *L. pneumophila* is able to replicate within both professional phagocytes from humans and mice, in addition to human lung epithelial cells that are not professionally phagocytic.

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3.2 Introduction

L. pneumophila is a facultative intracellular bacterial pathogen that replicates in human monocytes and alveolar macrophages (188, 250). The role of these cells is to destroy foreign invading antigens. Legionnaires' disease is a severe and often fatal form of pneumonia. During Legionnaires' disease, the organism is able to circumvent the normal destructive processes of macrophages and not only survive but replicate within these cells that evolution "designed" to eradicate such agents as *Legionella*.

Following recognition of the organism in 1977 (233), Rodgers *et al.* (289) demonstrated by electron microscopy that *L. pneumophila* functioned as a facultative intracellular pathogen replicating within host lung cells. Horwitz and Silverstein (188) initially described the interaction of *L. pneumophila* with human peripheral blood monocytes and since then a variety of human and animal cells have been shown to support the intracellular replication of the pathogen (258, 267, 286, 370). However, *L. pneumophila* is unable to replicate within PMNLs (186, 258). The alveolar macrophage is the primary cell infected; therefore, macrophages serve as the logical target cell to investigate the pathogenesis of *L. pneumophila* infection at the cellular level. As alveolar macrophages are difficult to obtain from humans or mice, alternative cells are required to study the pathogenic processes at the cellular level.

For bacterial infection to proceed, pathogens must first bind to their

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host cells (24). In the opsonin-dependent system, organisms are pre-coated with specific antibody and bind to phagocytic cells through Fc receptors. Two mechanisms of attachment have been described for *L. pneumophila;* opsonin-dependent (26, 266) and opsonin-independent (155, 154, 286). In opsonin-mediated adherence, complement fragments C3b and C3bi coat the organism prior to binding to the surfaces of monocytes via CR1 and CR3 receptors (26). C3bi is deposited on the major outer membrane protein (MOMP) of *L. pneumophila* and the MOMP/C3bi complex binds to CR3 on human monocytes (26). The opsonin-independent mechanism of attachment does not involve specific antibody or complement and the MOMP may facilitate the attachment of *L. pneumophila* to U-937 cells in this system (see Section 2).

Complement-mediated binding is followed by internalization of *Legionella* organisms via a process termed coiling phagocytosis (182). Internalized organisms are contained within banded enclosures (258). Phagosomes containing organisms then become ribosome-lined and are in close association with mitochondria (179, 258). It is not understood why this organelle recruitment should occur, however it appears to be necessary for intracellular replication of *L. pneumophila* (179). The survival of *L. pneumophila* within mononuclear cells depends on the inhibition of phagosome-lysosome fusion and acidification of the phagosome (181, 185). The precise mechanism of phagosome-lysosome inhibition has not been

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elucidated and is not well understood. However, recently, it has been shown that the *dot* (defective for organelle trafficking)/*icm* (intracellular multiplication) genes play a role in this process and are required for intracellular survival of *L. pneumophila* within the phagosome (297, 271). Through this inhibition, the normal degradative process that would follow to destroy foreign material is halted and unabated intracellular replication of the organism ensues. Following intracellular multiplication, *L. pneumophila* is released from the phagosome into the cytoplasm of the host cell and eventually escapes from the cell to the extracellular milieu to continue disease by infecting neighboring cells. The precise mechanism of escape has not been elucidated; however, cells rupture and are lysed with release of the bacteria. Gao *et al.* (143) have indicated that release of *L. pneumophila* may be preceded by macrophage apoptosis which results in lysis of infected cells. In addition, the major secretory protein of *L. pneumophila* may play a role in this process.

The terminal line in constitutive defense in the lung is the alveolar macrophage, the target cell of *L. pneumophila*. The intracellular nature of *L. pneumophila* in these cells aids organism survival by protecting it from the humoral branch of the immune system, thus, recovery from Legionnaires' disease relies on the cell-mediated branch of the immune system. The guinea-pig has been accepted as a model for human Legionnaires' disease; however, an ideal cell model for the study of legionellosis is not as readily

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available. Guinea pig alveolar macrophages, human peripheral monocytes, U-937 cells and HL-60 have all been used to investigate the microbiology and pathogenesis of *L. pneumophila* (102, 154, 190, 224, 267). In addition, these cells are not ideal as they only represent some of the normal activities of human alveolar macrophages *in vivo*; thus, it may be necessary to use more than one cell type to delineate the attachment and intracellular replication process for *L. pneumophila*. Early studies delineated the initial recognition of the bacteria by host cells in a complement-mediated receptor binding fashion (266). More recently, an attachment process that occurs in the absence of opsonins, a complement-independent mechanism, has been identified (155, 286). The recognition event, whether opsonin-mediated or opsonin-independent may determine the intracellular fate of the organism. It is possible that organisms entering macrophages via the complement route, trigger the normal killing process of the cell as has been shown for many pathogens (82, 93, 322), while the opsonin-independent attachment mechanism previously described, may not stimulate the oxidative events that would normally lead to the organism's death. If this were the case then the result would be fulminant intracellular replication.

3.3 Material and Methods

Reagent formulations and preparation along with detailed procedures are given in Appendix 1.

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3.3.1 Bacterial Cultivation

L. pneumophila stain Nottingham N7 was grown and maintained as previously described (see Section 2.3.1).

3.3.2 Eukaryotic Cell Growth and Maintenance

U-937 cells, A549 cells and thioglycollate-elicited A/J murine peritoneal macrophages were grown and maintained as previously described (see Section 2.3.2).

3.3.3 Intracellular Replication Assay

Intracellular replication and release of *L. pneumophila* was assessed in U-937, A549 and thioglycollate-elicited peritoneal macrophages from A/J mice. Monolayers of eukaryotic cells in 24-well plates were washed three times with HBSS to remove non-adherent cells. *L. pneumophila* ($5x10^7$ CFU/ml) in serum-free HBSS was added to these monolayers at a MOI of 100 and were allowed to adhere to cells for 1 h at 37°C in 5 % CO_2. Following the 1 h organism-attachment period, supernatant fluids were removed and cells were washed three times with HBSS to remove unbound bacteria. The three washings along with the supernates were collected and pooled for each cell type. Serial 10-fold dilutions of these pooled samples were made in 1 % (w/v) peptone and duplicate samples of 25 µl were plated on BCYE-α agar. A 1 ml volume of either RPMI-1640 for U-937 cells and A/J peritoneal macrophages

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or Ham's FK12 for A549 cells supplemented with 10% heat-inactivated FBS was then added to all wells. At 6, 12, 24, 36, 48 and 72 h post-adherence cell monolayers were assessed by VBCC counts for both intracellular as well as extracellular bacteria.

3.3.4 Viable bacterial cell colony (VBCC) counts

Supernatant fluids were collected, cells were washed three times with HBSS and added to the supernatant fluids at which time 1 ml sterile water was added to cell monolayers for 20 min. Cell were lysed by vigorous aspiration and expulsion through a sterile pasteur pipette. A 100 µl aliquot from each of the supernatant fluids plus wash samples as well as each lysate were serially diluted 10-fold in 1 % (w/v) peptone and duplicate samples of 25 µl were plated on BCYE-α agar to enumerate both intracellular and released legionellae from U-937, A549 and A/J thioglycollate-elicited peritoneal macrophages.

3.4 Results

L. pneumophila adhered readily to U-937 cells within 1 h. VBCC count data indicated that approximately $5x10^5$ bacteria (approximately 1 % of the inoculum) had attached to U-937 cells during the 1 h incubation period, corresponding to 1 bacteria bound to each U-937 cell (Figure 3.1). This period was followed by one of rapid intracellular replication for 36 h. A 10-fold

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increase in the intracellular replication of *L. pneumophila* within U-937 cells was observed from 12 to 36 h. Lysis of U-937 cells between 36 and 48 h was seen microscopically and this lysis resulted in the release of progeny *L. pneumophila*. A similar profile of *L. pneumophila* replication was observed for A549 cells. Attachment of approximately $5.5x10^5$ bacteria (approximately 1.1 % of the inoculum) occurred within 1 h resulting in a ratio of 1.1 bacteria bound to each A549 cell (Figure 3.2). Intracellular replication, from 24 to 36 h, resulted in a 10-fold increase in the numbers of *L. pneumophila* within A549 cells. *L. pneumophila* growth in cells effected lysis of host cells between 36 and 48 h releasing progeny as indicated by the rise in the numbers of extracellular bacteria present in supernatant fluids. Thioglycollate-elicited peritoneal macrophages from A/J mice supported the intracellular replication of *L. pneumophila* and resulted in a 12-fold increase in the numbers of bacteria within host cells from 12 to 36 h (Figure 3.3). Approximately $3.1x10^5$ bacteria (approximately 0.6 % of the inoculum) initially bound to host cells following the 1 h incubation period, corresponding to 0.622 bacteria bound to each A/J peritoneal macrophage. Lysis occurred between 36 and 48 h releasing bacteria into the extracellular milieu. VBCC counts of washed samples (extracellular bacteria) and cell lysates (intracellular bacteria) demonstrated maximal intracellular *Legionella* numbers at 36 h of infection for all cell types investigated (Figures 3.1, 3.2 and 3.3). As intracellular replication continued toward later stages of infection, an increase in the

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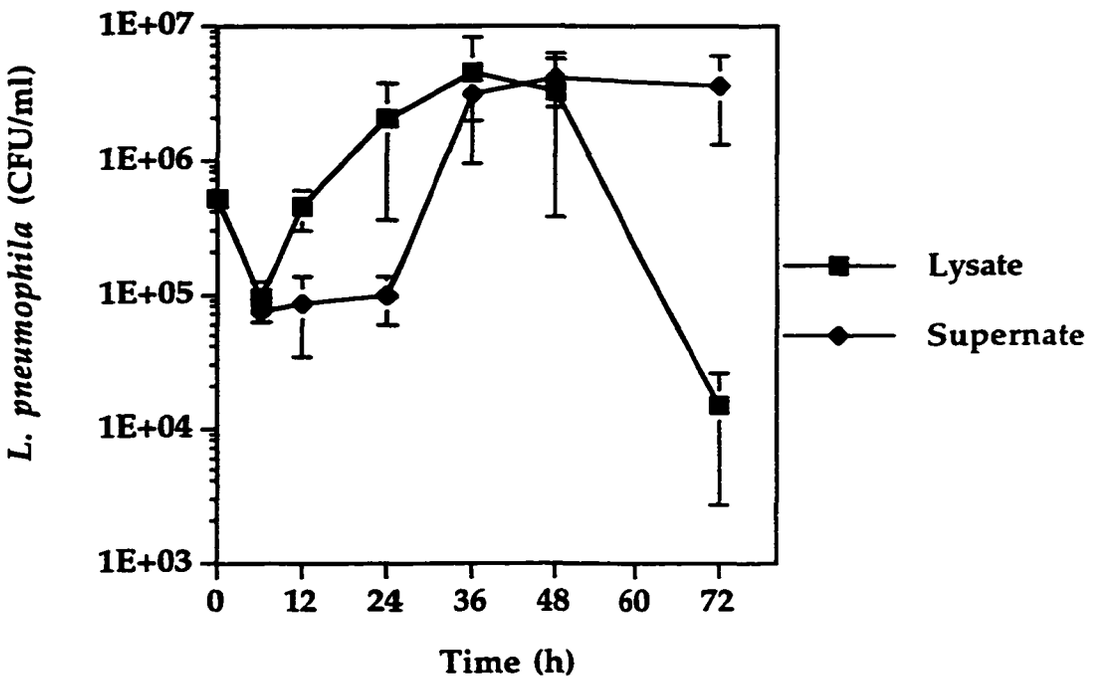

Figure 3.1: Attachment and intracellular replication of *L. pneumophila* within U-937 cells as assayed by VBCC counts. U-937 cells were seeded at $5x10^5$ cells/well and infected at a multiplicity of infection of 100 for 1 h. Intracellular replication and release of *L. pneumophila* are shown. Results are expressed as means ± standard deviations from five separate experiments each of which were conducted in triplicate (i.e. 15 data points per column).

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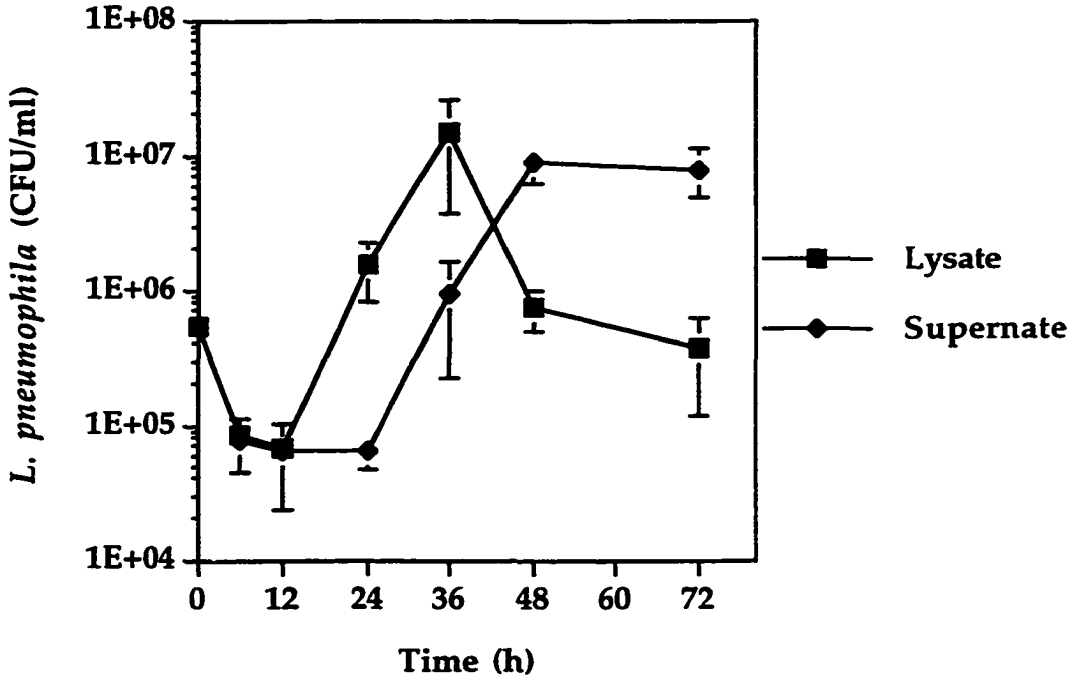

Figure 3.2: Attachment and intracellular replication of *L. pneumophila* within A549 cells as assayed by VBCC counts. A549 cells were seeded at $5x10^5$ cells/well and infected at a multiplicity of infection of 100 for 1 h. Intracellular replication and release of *L. pneumophila* are shown. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate (i.e. 9 data points per column).

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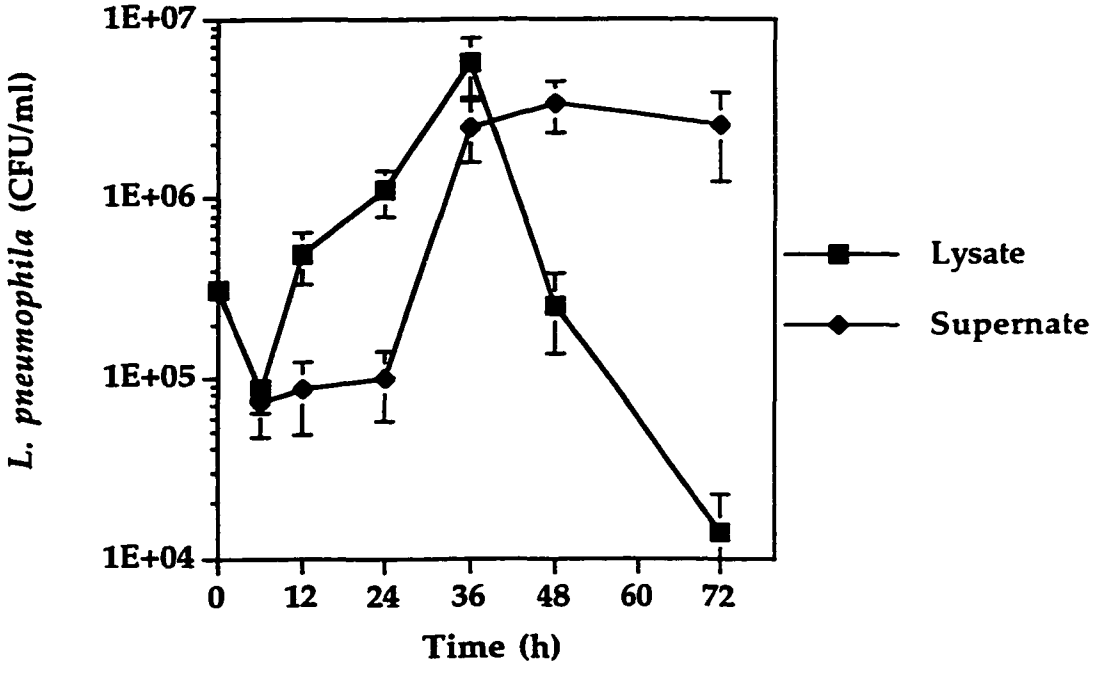

Figure 3.3: Attachment and intracellular replication of *L. pneumophila* within thioglycollate-elicited peritoneal macrophages from A/J mice as assayed by VBCC counts. Macrophages were seeded at $5x10^5$ cells/well and infected at a multiplicity of infection of 100 for 1 h. Intracellular replication and release of *L. pneumophila* are shown. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate (i.e. 9 data points per column).

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number of bacteria was found in the extracellular environment with a simultaneous decrease in the number of intracellular organisms. Further experiments demonstrated that *L. pneumophila* did not replicate extracellularly in HBSS, RPMI-1640 with or without 10 % heat inactivated FBS or in HAM's FK12 with or without 10 % heat inactivated FBS either in the presence or absence of host cells.

3.5 Discussion

L. pneumophila infections constitute a significant medical problem especially among immunocompromised individuals. It is critical that representative cell models are developed to study the infectious process of *L. pneumophila* at the cellular level of infection. The antimicrobial agents, erythromycin, ciprofloxacin, tetracycline and rifampin singularly or in combination have been used with varying success to control *Legionella* infections; however, individuals often succumb to Legionnaires' disease despite aggressive antibacterial therapy and mortality rates as high as 40 % have been associated with Legionnaires' disease (131, 163). Defining the specific interactions between *L. pneumophila* and cells they infect is necessary for the development of alternative therapeutic strategies to augment current antimicrobial treatment and/or prevention regimes.

The binding process of pathogen to host cell is complex and is most likely multifactorial. *L. pneumophila* organisms are able to bind to and

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replicate within alveolar macrophages; thus, they are protected from the host's humoral immune defenses. Therefore, mediating a cell-mediated immune response is critical to recovery. Several transformed cells have been proposed as potential cell models to study *Legionella*-host interactions but it is the U-937 cell that has been used most frequently for this purpose (1, 143, 144, 153, 154, 217, 267, 269, 286). This is particularly relevant as these cells can be differentiated to macrophage-like cells expressing the complement receptors CR3 and CR4 at levels similar to alveolar macrophages.

In this study the ability of *L. pneumophila* to attach to and replicate within U-937 and A549 cells as well as in thioglycollate-elicited peritoneal macrophages from A/J mice was evaluated in an opsonin-independent system of infection. Since A549 cells are not professionally phagocytic and do not possess complement and Fc receptors normally found on the surface of mononuclear cells, *L. pneumophila* uses other mechanisms to attach to and infect these cells. Furthermore, in the environment, legionellae colonize and infect fresh water amoebae in the absence of opsonic components (18). In addition, Reynolds and Newball (277) have demonstrated that the distal bronchioles and the alveolar space of the human lung contain very low levels of complement. Thus, the study of *L. pneumophila* infection of host cells with and without Fc and other mononuclear cell receptors both in the presence and in the absence of opsonins is of critical importance in developing a rationale for defining the initial recognition and colonization

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events of host cells by *L. pneumophila.*

Studies by Horwitz *et al.* (182) have demonstrated that *L. pneumophila,* in the presence of complement components, enters mononuclear cells by a process termed coiling phagocytosis in which long pseudopods coil around the organism as it is internalized. However, these workers also noted that not all *L. pneumophila* are taken up by this process, indicating either organism-based differences were occurring or alternatively two different mechanisms of uptake were taking place simultaneously. Once internalized, the organism resides within tight-fitting phagosomes that are lined with host cell ribosomes and mitochondria (179). Inhibition of phagosome-lysosome fusion is crucial to organism survival and subsequent intracellular replication within host cells (7, 181, 185, 258, 349).

In the present study *L. pneumophila* was able to adhere to and replicate within cells that are professionally phagocytic (U-937 and A/J macrophages) as well as in cells that are not (A549) in an opsonin-independent manner. The replication and lytic profiles were similar for all cell types investigated. Following organism attachment a decrease in numbers of *L. pneumophila* were noted for all cell types from initial attachment to 6 h post-infection. This decrease in CFU/ml may represent initial organism killing by the host cells. It is possible that the pathogen was then able to overcome host cell defenses and replicate within these cells eventually leading to cell lysis between 36 to 48 post-infection.

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This study demonstrates that opsonin-independent processes may be necessary for the initial interaction and establishment of cellular infection and that complement-mediated processes may follow only after cellular destruction and inflammation proceed. Furthermore, it has been demonstrated that complement, in the absence of specific antibody, does not promote the adherence of *L. pneumophila* to U-937 cells, guinea pig alveolar macrophages or J774 mouse macrophages (190). The work in the present study coupled with that of Husmann and Johnson (190) indicate that an opsonin-independent attachment mechanism may exist for *Legionella* and that this process may dictate the outcome of infection. In addition, the present work further demonstrates the usefulness of U-937 cells and peritoneal macrophages from A/J mice as models to study *L. pneumophila* infections of cells in the host. A549 cells, which are characterized as type II pneumocytes, line the alveolus of the lung and may contribute to the overall pathogenesis of infection by supporting *L. pneumophila* growth *in vivo* in a manner similar to that *in vitro*. Further work to identify the interaction process of *L. pneumophila* with cells that reside in the lung, other than the alveolar macrophage, will aide in delineating specific and key pathogenic mechanisms for establishing disease. Once the host-bacteria relationship can be dissected it will facilitate the discovery of alternative therapies to assist in resolving Legionnaires' disease. These three cells may prove crucial to that process.

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CHAPTER IV

IN VITRO CYTOKINE PRODUCTION BY U-937 CELLS CHALLENGED WITH *LEGIONELLA PNEUMOPHILA*

4.1 Abstract

Cytokines are immune modulating protein molecules of low molecular weight produced by various cells that regulate the intensity and duration of an immune response to an antigen. A major area of research that has received considerable attention is understanding the role cytokines play in modulating the immune system during infection with many obligate and facultative intracellular pathogens. There is limited information regarding the production of cytokines during infection with *Legionella pneumophila*. In this study the production of the proinflammatory cytokines, interleukin (IL)-1β and tumor necrosis factor (TNF)-α, the chemokine, IL-8 and the immune regulating cytokines IL-10, IL-12 and interferon (IFN)-γ from macrophage-like U-937 cells in response to *L. pneumophila* infection was investigated. Infection of U-937 cells, stimulated into the adherent macrophage-like form with phorbol esters, by *L. pneumophila* elicited the production of IL-1β and IL-8, while TNF-α, IL-12 and IFN-γ were not

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stimulated during infection. Interestingly, U-937 cells challenged with *L. pneumophila* produced decreased levels of IL-10 as compared to uninfected controls. The effect of *L. pneumophila* viability on the induction of cytokines produced by U-937 cells was assessed by challenging the cells with either live, heat-killed or formalin-fixed *L. pneumophila.* Inoculation of U-937 cells with either heat killed or formalin fixed *L. pneumophila* elicited significantly greater levels of IL-8 and IL-10 as compared to cells infected with live *L. pneumophila;* however, killed organisms failed to elicit IL-1β, TNF-α, IL-12 and IFN-γ from U-937 cells. These results suggest that *L. pneumophila* infection elicits specific cytokines from affected cells, and that the normal cytokine response of macrophages is profoundly affected following *Legionella* infection.

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4.2 Introduction

The facultative intracellular pathogen *Legionella pneumophila* is the causative agent of Legionnaires' disease, an acute multifocal necrotizing pneumonia. When the organism is inhaled by humans it descends into the lower respiratory tract and parasitizes alveolar macrophages (250). Studies have demonstrated the importance of the macrophage-*L. pneumophila* interaction that permits *L. pneumophila* to persist in the lung (34, 57, 86, 312, 311) . Furthermore, it is widely accepted that a cell-mediated host response is crucial to the resolution of Legionnaires' disease (41, 40, 77, 137, 253, 315). Investigators have used a variety of *in vitro* models to study cell infection by *L. pneumophila* and those studied include cells of human and non-human origin as well as protozoa (2, 3, 18, 122, 120, 258, 267, 370). Based on the nature of the disease process and the observation that *L. pneumophila* is an intracellular pathogen, investigations have begun to investigate the interactions of *L. pneumophila* with the host's immune system. In light of the high mortality rate associated with Legionnaires' disease and the lack of resolution often associated with appropriately treated disease, alternative therapeutic strategies are required. Vaccination, immunotherapy and cytokine therapy have been suggested to augment current antibiotic treatments. However, target bacterial molecules for use in vaccines and precise protocols for immunological intervention are not currently available. One potential avenue involves the use of naturally occurring host molecules

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or response systems, such as cytokines, to amplify the crucial host response to combat disease (140, 134, 214, 248). Cytokines are a class of soluble low molecular weight glycoproteins that mediate cell-to-cell communication during an immune response (10, 216). Membrane-bound forms have also been described for many cytokines. Cytokines comprise several groups of molecules including interleukins, lymphokines, monokines, interferons, colony-stimulating factors and a variety of other proteins (9, 10, 81). Initially it was thought that these molecules were produced exclusively by leukocytes, lymphocytes, and monocytes; however, epithelial and endothelial cells can secrete a range of these as well (216). Cytokines exhibit synergistic, antagonistic, redundant and pleiotropic properties. Through these attributes, this diverse group of molecules mediates, maintains, regulates and dictates the intensity and duration of the immune response in a coordinated, interactive fashion by modulating the activity of immune effector cells (134, 216). The effects of cytokines on target cells are exerted by binding to specific membrane bound cytokine receptors which function as ligand binding and signal transduction moieties (239). The cellular consequences of ligand binding are diverse and involve the induction of transcription of cytokine coding genes, as well as a diverse set of responses associated with cellular proliferation and differentiation (81, 239). It has been suggested that the sharing of receptor subunits may, in part, reflect the redundant functions of cytokines (216, 239).

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Functions of immune cells are finely coordinated to ensure an adequate response to an antigenic stimulus, and part of this coordination is mediated by secreted cytokines. Cytokines regulate the immune response by stimulating or inhibiting the proliferation of various cells or by influencing the secretion of antibodies or other cytokines (81, 216). The development of an effective immune response involves lymphoid and inflammatory cells and other hematopoietic cells.

To date a detailed understanding of the host immune response to *L. pneumophila* has been hampered due to the lack of relevant systems to study infection. Several investigators have used *in vitro* systems to define the production of cytokines elicited by *L. pneumophila* or its component antigens during infection of cells *in vitro*. Increased levels have been described for mRNA specific for IL-1-α, IL-1β, IL-6, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) following *L. pneumophila* attachment to murine peritoneal macrophages (371, 367). In addition, binding of *L. pneumophila* to murine macrophages *in vitro* induces an increase in mRNA levels specific to the chemokines, macrophage inflammatory protein (MIP)-1β, MIP-2 and KC (367). *Legionella*, as well as purified antigens from this organism, serve to induce the proinflammatory cytokine, IL-1β from murine peritoneal, splenic and pulmonary macrophages as well as from human peripheral blood monocytes (358). Matsiota *et al.* (230) showed that *L.*

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pneumophila infection of human monocytes resulted in a low level induction of TNF-α *in vitro*. Park and Skerrett (264) have shown that human monocytes infected with *L. pneumophila* secrete higher levels of IL-10 than similarly infected alveolar macrophages *in vitro*. TNF-α and IFN-γ are also produced by lymphocytes challenged with *L. pneumophila in vitro* (203).

It has been proposed that resolution of *Legionella* infections is facilitated by activation of macrophages and these subsequently resist *Legionella* infection. Initial reports indicated that treatment of monocytes with IFN-γ was able to limit intracellular replication of *L. pneumophila* in human peripheral blood monocytes (31, 30, 65). This observation was subsequently confirmed using human alveolar macrophages (194, 250, 249). IFN-γ downregulates the function of complement receptors that mediate phagocytosis of *L. pneumophila*; thus, activated mononuclear phagocytes inhibit *L. pneumophila* multiplication by phagocytozing 50 % fewer *L. pneumophila* (65, 186, 303). This restricts access of the bacteria to the intracellular milieu required for replication. In addition, IFN-γ activated monocytes and macrophages decrease the multiplication rate of *L. pneumophila* that are internalized by reducing iron availability. This bacteriostatic effect induced by IFN-γ correlates with a 73 % down-regulation of transferrin receptors (TfR) on the macrophage surface and an 83 % decrease in the concentration of intracellular ferritin (65). Hamilton *et al.* (161) also

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found a threefold reduction in TfR binding sites in thioglycollate-elicited murine macrophages treated with IFN-γ. The resultant net decrease in the concentration of intracellular iron restricts *L. pneumophila* growth within macrophages. Furthermore, it has been suggested that the mechanism of protection afforded by IFN-γ pretreatment of macrophages is mediated, in part, by TNF-α. Indeed, pretreatment of human monocytes with IFN-γ prior to *L. pneumophila* infection results in an increased TNF-α response (230). Further studies suggest that the protection afforded by IFN-γ treatment can be overcome by subsequent exposure of alveolar macrophages or monocytes to the immunoinhibitory cytokine, IL-10, and this results in active intracellular replication of *L. pneumophila* (264). Data concerning the secretion of specific cytokines induced in cells by *L. pneumophila* infection are limited. A broad study has yet to be performed to investigate the ability of *L. pneumophila* to induce various cytokines during intracellular replication. Table 4.1 depictes the cytokines that were investigated in the present study and they comprise proinflamatory cytokines, a chemokine and immune-regulating cytokines. The present study serves to increase the current knowledge regarding the cytokines that may be crucial to disease development during *L. pneumophila* infection.

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Table 4.1. Cytokines investigated during present study.

Cytokine	Cellular source(s)	Biologic actions
Interleukin-1β (IL-1β) 17.5 kDa 153 aa	Monocytes, macrophages, neutrophils, endothelial cells epithelial cells, fibroblasts, T and B lymphocytes, NK cells, keratinocytes, dendritic cells	Induces acute phase response; increases IL-2 receptor expression; activates NK cells; promotes B and T lymphocyte proliferation; endogenous pyrogen
Tumor necrosis factor-α (TNF-α) 17 kDa 157 aa	Monocytes, macrophages, T and B-cells, NK cells, neutrophils, astrocytes, endothelial cells, smooth muscle cells, mast cells, fibroblasts	Multiple biological properties; antitumor and antimicrobial activities; cytolytic effects; acts synergistically with IFN-γ; enhances phagocytosis of PMNLs; activates macrophages, lymphocytes,neutrophils; endogenous pyrogen; induces fever and shock-like symptoms; enhances CD14 expression on PMNLs; induces expression of adhesion molecules (VCAM-1 and E-selectin)
Interleukin-8 (IL-8) 8-10 kDa 69-77 aa	Monocytes, macrophages, neutrophils, T-cells, fibroblasts, keratinocytes, endothelial and epithelial cells, astrocytes, smooth muscle cells	Activates neutrophils; T lymphocytes, basophils; induces respiratory burst in neutrophils; adherence of peripheral mononuclear cells to endothelial cells via enhanced expression of CD11/CD18; upregulation of complement receptors CR1 and CR3 in human neutrophils

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Interleukin-10 (IL-10) 18 kDa 178 aa	Human Th0, Th1, Th2, murine Th2, macrophages, activated monocytes, T and B lymphocytes, Keratinocytes, mast cells	Pleiotropic cytokine; Suppresses macrophage function; inhibits production of IFN-γ by Th1 cells, antigen presenting cell function and IL-1, IL-6 and TNF-α from macrophages; stimulates B lymphocyte production and immunoglobulin secretion
Interleukin-12 (IL-12) 35 and 40 kDa p35 (219 aa), p40 (328 aa)	Monocytes, macrophages, NK cell, B-cells, keratinocytes, Langerhans cells	Activates CD4 T cells; Th1 induction and maturation; induces IFN-γ production by NK and T-cells; NK cell induction; enhances cytolytic activity of NK cells and macrophages
Interferon-γ (IFN-γ) 17-25 kDa 166 aa, 23 aa	T lymphocytes, NK cells, endothelial cells, dendritic cells	Activates macrophages; NK cells, Tc and B cells; enhances macrophage killing of intracellular pathogens; increases surface expression of MHC class I on various cell types; increases surface expression of MHC class II antigens on antigen-presenting cells; induces de novo expression of MHC class II antigens on various cells; induces IFN-γ mRNA

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4.3 Materials and Methods

Reagent formulations and preparation along with detailed procedures are given in Appendix 1.

4.3.1 Bacterial Cultivation

L. pneumophila stain Nottingham N7 was grown and maintained as previously described (see Section 2.3.1). *L. pneumophila* ($5x10^7$ CFU/ml) were heat killed by boiling for 10 min in HBSS. In addition, aliquots of heat killed, bacteria were centrifuged for 10 min at 6,000 x g to obtain bacterial pellets and supernatant fluids. Bacterial pellets were washed three times by centrifugation with HBSS and resuspended in fresh HBSS prior to infection assays. The supernatant fluid was added directly to host cells. Further aliquots of *L. pneumophila* were fixed in 10% buffered formalin for 30 min. Bacteria were washed three times by centrifugation in HBSS to remove formalin. All viable bacterial inocula were confirmed by CFU/ml determination as assayed by VBCC counts. In addition, heat-killed and formalin fixed preparations were evaluated for viability by VBCC counts.

4.3.2 U-937 cell Growth and Maintenance

U-937 cells were grown, maintained and phorbol ester treated as previously described (see Section 2.3.2).

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4.3.3 *L. pneumophila* Infection of U-937 Cells

Monolayers of U-937 cells in 24-well plates were washed three times with HBSS to remove non-adherent cells and serum components. Live *L. pneumophila* at $5x10^7$ CFU/ml or an identical number of heat killed or formalin fixed *L. pneumophila* in serum-free HBSS were added to the monolayers of U-937 cells (multiplicity of infection of 100) and allowed to adhere for 1 h at 37°C with 5% CO_2. Following the 1 h *L. pneumophila*-host cell attachment period, culture supernatant fluids were collected, centrifuged and filtered through a 0.2 μm filter to remove unbound bacteria and stored for cytokine analysis. *L. pneumophila*-infected U-937 cell monolayers were washed three times with HBSS to remove all unbound bacteria. Washing efficiency was determined by plating the final wash. A 1 ml volume of RPMI-1640 supplemented with 10 % heat-inactivated FBS was added to all wells and the U-937 cell monolayers were incubated at 37°C. At 6, 12, 24, 36 and 48 h post-adherence supernatant fluids were collected, centrifuged, filtered and stored frozen for cytokine analysis. In addition, bacterial burden in infected U-937 cells was determined by VBCC counts (see section 3.3.4). All collected supernatant fluids were centrifuged at 6,000 x g and stored at -70°C for assay using human cytokine-specific ELISA kits (Biosource International, Camarillo, CA.).

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4.3.4 Determination of Cytokine Levels from U-937 Cell Cultures

Supernatant fluids from *L. pneumophila* challenged U-937 cells were thawed and assayed for IL-1β, TNF-α, IL-8, IL-10, IL-12 and IFN-γ using human cytokine-specific ELISA kits (Biosource International, Camarillo, CA.). ELISA was performed as per manufacture's instructions and optical densities were read on a Bio-Rad microplate reader at 450 nm. The concentration of each cytokine in the culture preparations were calculated from standard curves generated during each assay.

4.3.5 Statistical Analysis

The nonparametric Mann-Whitney test was performed to analyze differences in levels of cytokines between control, uninoculated monolayers, and cells that had been exposed to viable or killed legionellae using the statistical analysis software, InStat (GraphPad Software Version 2.00, San Diego, CA.) on a Power Macintosh Performa 6214CD. A p value of less than 0.05 was considered significant.

4.4 Results

Viable *L. pneumophila* adhered readily to U-937 cells and maximum binding was achieved within 1 h. This was followed by rapid intracellular replication over the following 36 h. Lysis of U-937 cells occurred between 36 and 48 h and resulted in the release of progeny *L. pneumophila* (see Figure

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3.1).

The production of IL-1β, TNF-α, IL-8, IL-10, IL-12 and IFN-γ from uninfected U-937 cells and from cells challenged with either live, heat-killed or formalin-fixed *L. pneumophila* was investigated. The proinflammatory cytokine, IL-1β was induced by *L. pneumophila* infection and this reached maximum levels by 48 h post-infection (1004 ± 39 pg/ml) (Figure 4.1) while TNF-α was not elicited by *L. pneumophila* challenge of U-937 cells (Figure 4.2). IL-8, a chemokine primarily involved with neutrophil priming, was elicited at low levels during early infection (6 h) and increased steadily to maximum levels by 48 h (12,300 ± 430 pg/ml) (Figure 4.3). As seen in Figure 4.5, U-937 cells infected with *L. pneumophila* showed a significant decrease in levels of IL-10 during early intracellular replication as compared with controls, indicating that infection may have resulted in down-regulation of IL-10 production. In this study minimum levels of IL-12 (Figure 4.7) and IFN-γ (Figure 4.8) were detected in supernatant fluids from either infected or uninfected U-937 cells.

To determine whether the viability of *L. pneumophila* was critical for eliciting individual cytokine responses from *Legionella*-infected U-937 monolayers, cells were challenged with a similar inocula of either live, heat-killed or formalin-fixed *L. pneumophila*. IL-1β (Figure 4.1) and TNF-α (Figure 4.2) were not detected in supernatant fluids of U-937 cells following

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challenge with either heat-killed or formalin-fixed *L. pneumophila* ($p < 0.005$ as compared with live *L. pneumophila* challenge for IL-1β). Heat-killed *L. pneumophila* elicited high levels of IL-8 in the supernatant fluids from U-937 cells within 6 h and these levels were maintained to significantly higher levels than in those fluids from cells infected with live *L. pneumophila* ($p < 0.006$ and 0.03) (Figure 4.3). To assess whether the molecule(s) responsible for the increased levels of IL-8 following treatment of cells with heat-killed *L. pneumophila* were located on the surface of the organisms or were being secreted by *L. pneumophila,* U-937 cells were challenged with either the bacterial pellet or the supernatant fluid of *L. pneumophila* preparations after heat inactivation. As shown in Figure 4.4 the bacterial pellet induced significantly greater levels of IL-8 as compared to the supernatant fluid. However, the supernatant fluid alone also resulted in a greater level of IL-8 produced from U-937 cells as compared to live organism challenge. Formalin- fixed *L. pneumophila* resulted in significantly higher levels of IL-8 early during challenge and these levels remained steady over the 48 h period as compared to infection with live *L. pneumophila* (Figure 4.3). Culture of U-937 cells with either heat-killed or formalin-fixed *L. pneumophila* effected significantly higher levels of IL-10 detected in supernatant fluids compared with fluids from U-937 cells challenged with live *L. pneumophila* ($p < 0.03$ and 0.008) (Figure 4.5). To define where the cytokine inducing molecule(s) were located on *L. pneumophila* organisms, similar studies to those

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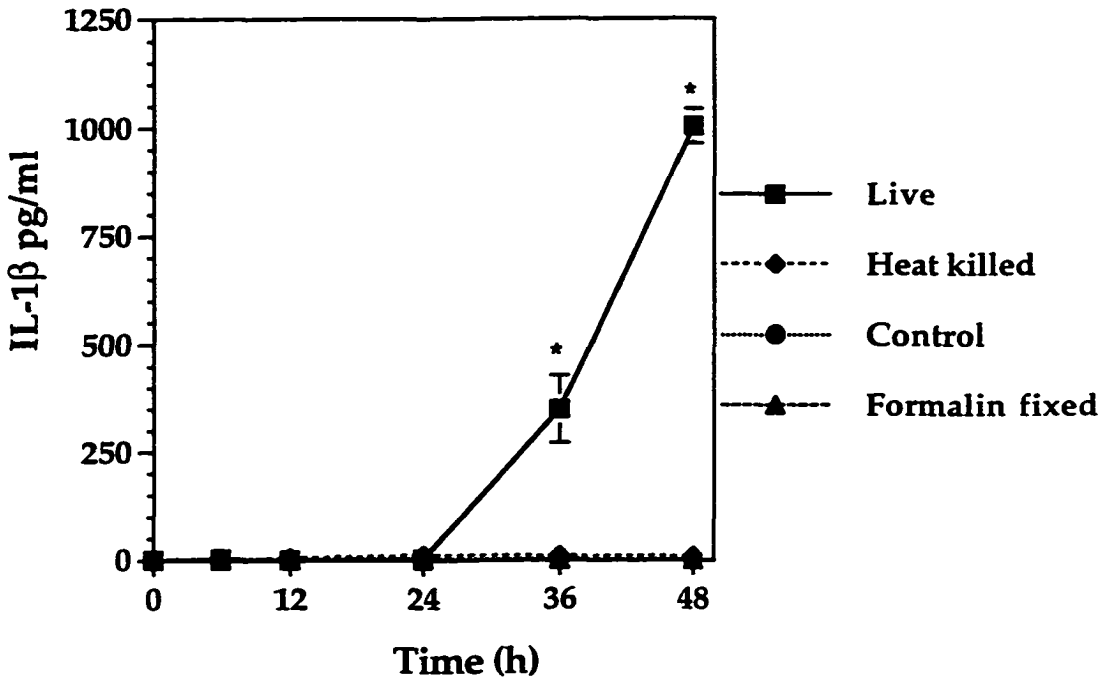

Figure 4.1: Interleukin-1β secretion from human macrophage-like U-937 cells challenged with live, heat-killed or formalin-fixed *L. pneumophila*. Control cultures did not receive *L. pneumophila*. Supernatant fluids were collected at the indicated time points during challenge and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate (i. e. 9 values/data point). $*p < 0.005$. Data for control and following inoculation with heat-killed or formalin-fixed organisms remained at base- line levels.

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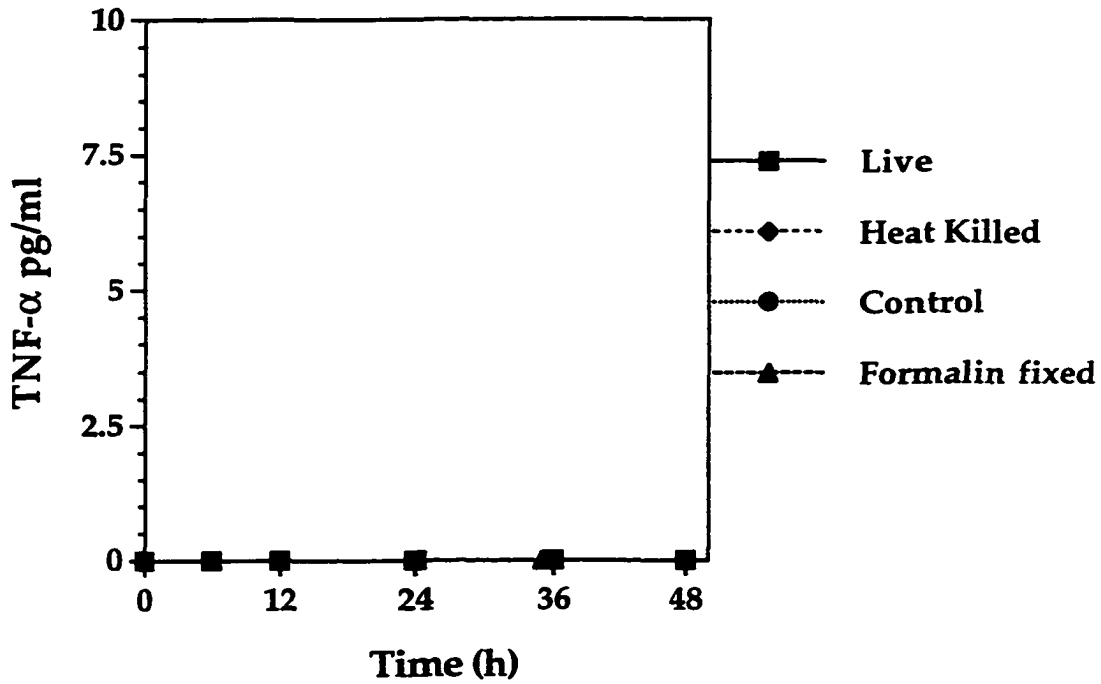

Figure 4.2: Tumor necrosis factor-α secretion from human macrophage-like U-937 cells challenged with live, heat-killed or formalin-fixed *L. pneumophila*. Control cultures did not receive *L. pneumophila*. Supernatant fluids were collected at the indicated time points during challenge and assayed for cytokine production by ELISA. Results are from three separate experiments each of which were conducted in triplicate (i. e. 9 values/data point). All figures remained at base-line levels throughout these experiments. Control positive assays and standard curves were normal.

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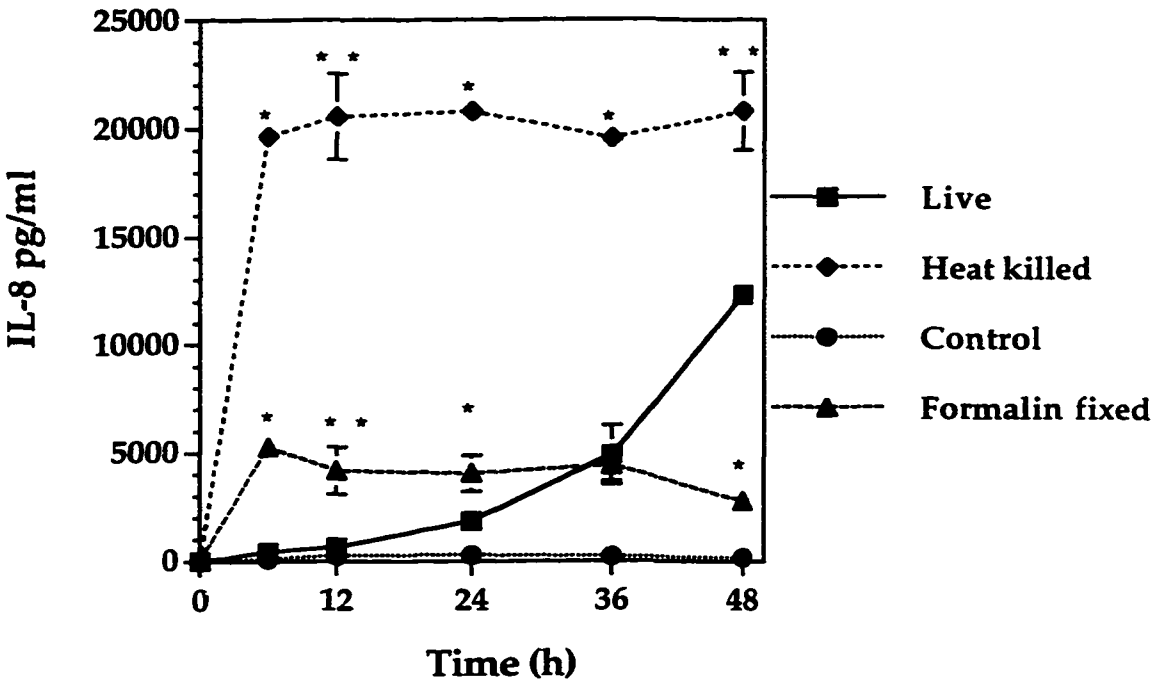

Figure 4.3: Interleukin-8 secretion from human macrophage-like U-937 cells challenged with live, heat-killed or formalin-fixed *L. pneumophila*. Control cultures did not receive *L. pneumophila*. Supernatant fluids were collected at the indicated time points during challenge and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate (i. e. 9 values/data point). $*p < 0.006$, $**p < 0.03$.

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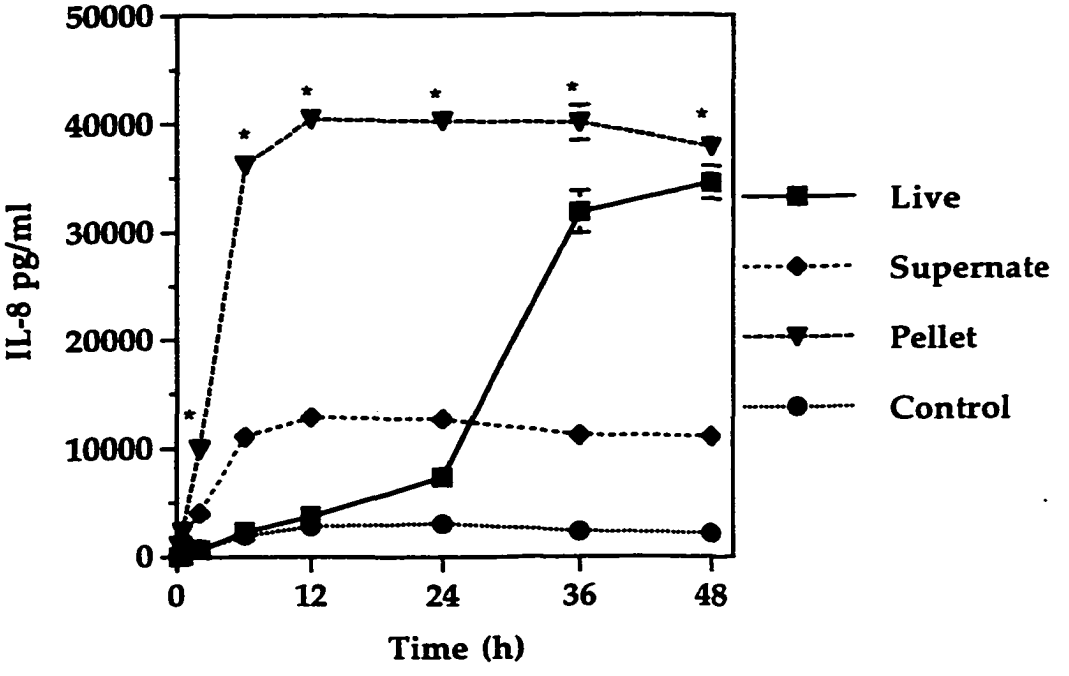

Figure 4.4: Interleukin-8 secretion from human macrophage-like U-937 cells challenged with live or heat-killed (supernatant fluid or bacterial pellet) *L. pneumophila*. Control cultures did not receive *L. pneumophila*. Supernatant fluids were collected at the indicated points during challenge and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate (i. e. 9 values/data point). $*p = 0.0286$.

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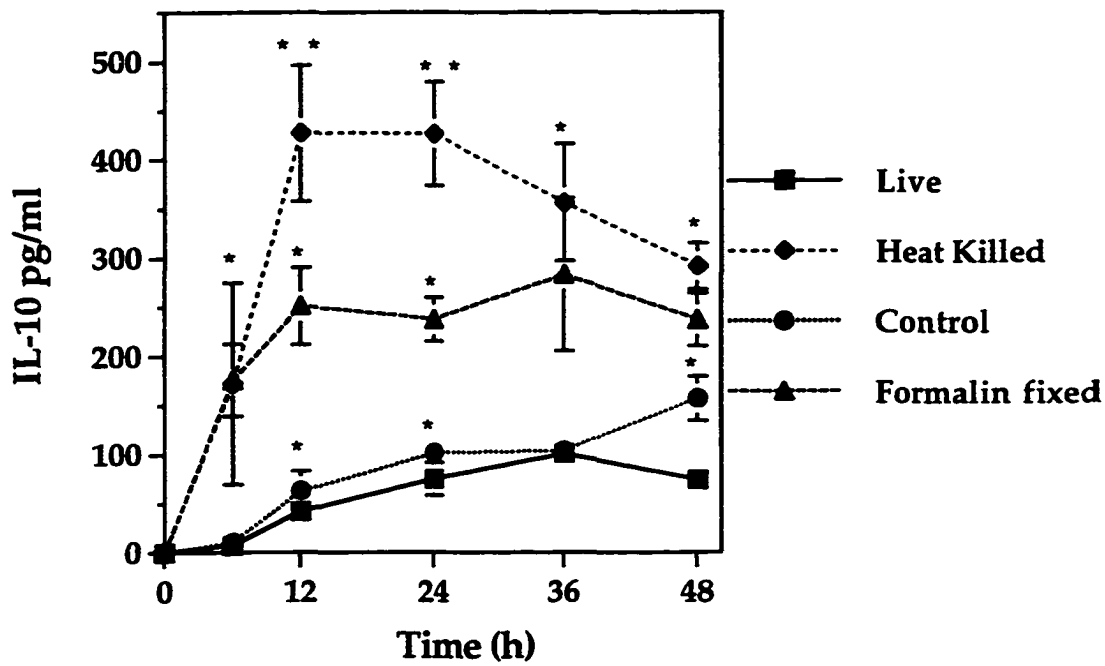

Figure 4.5: Interleukin-10 secretion from human macrophage-like U-937 cells challenged with live, heat-killed or formalin-fixed *L. pneumophila*. Control cultures did not receive *L. pneumophila*. Supernatant fluids were collected at the indicated time points during challenge and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate (i. e. 9 values/data point). $^{*}p < 0.03$, $^{**}p < 0.008$.

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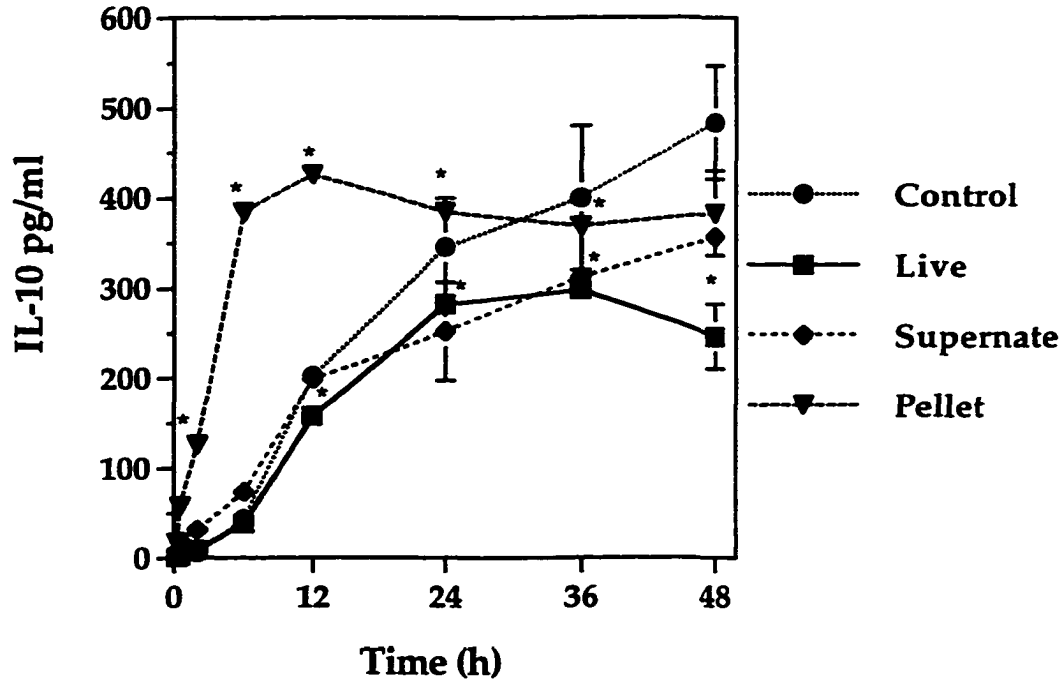

Figure 4.6: Interleukin-10 secretion from human macrophage-like U-937 cells challenged with live or heat-killed (supernatant fluid or bacterial pellet) *L. pneumophila*. Control cultures did not receive *L. pneumophila*. Supernatant fluids were collected at the indicated time points during challenge and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate (i. e. 9 values/data point). $*p = 0.0286$.

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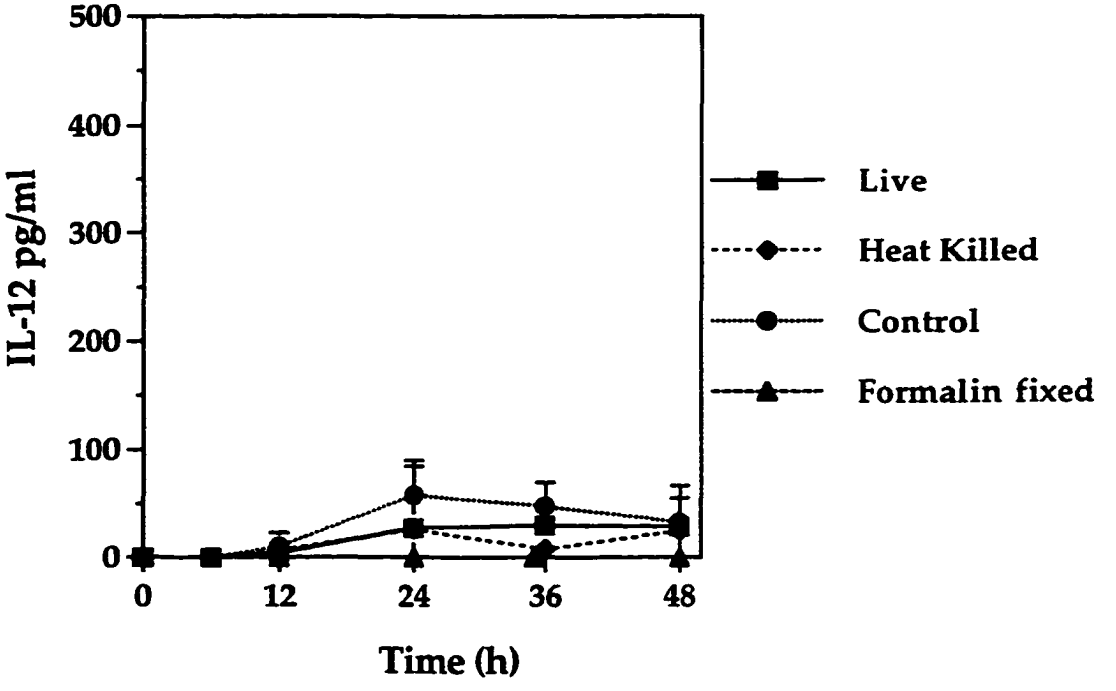

Figure 4.7: Interleukin-12 secretion from human macrophage-like U-937 cells challenged with live, heat-killed or formalin-fixed *L. pneumophila*. Control cultures did not receive *L. pneumophila*. Supernatant fluids were collected at the indicated time points during challenge and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate (i. e. 9 values/data point).

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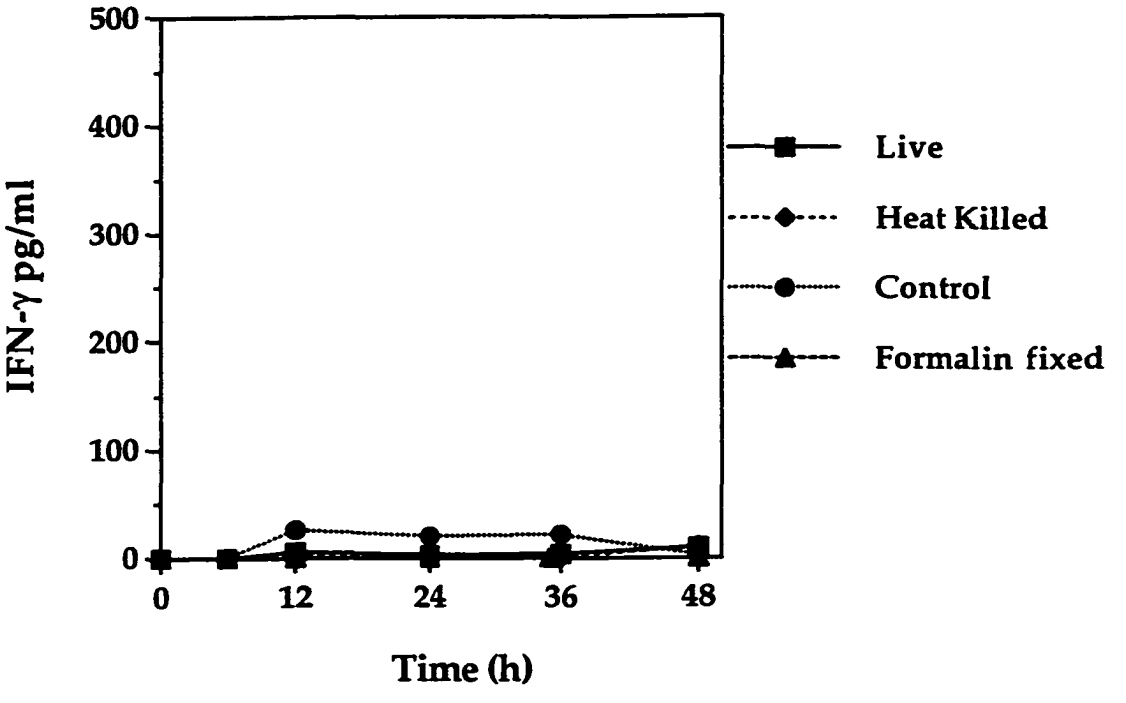

Figure 4.8: Interferon-γ secretion from human macrophage-like U-937 cells challenged with live, heat-killed or formalin-fixed *L. pneumophila*. Control cultures did not receive *L. pneumophila*. Supernatant fluids were collected at the indicated time points during challenge and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each of which were conducted in triplicate (i. e. 9 values/data point).

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conducted for IL-8 were performed for IL-10. Similar results were obtained (Figure 4.6). Significant levels of IL-12 and IFN-γ were not detected in supernatant fluids from U-937 cells challenged with either heat-killed or formalin-fixed *L. pneumophila* (Figure 4.7 and 4.8 respectively).

4.5 Discussion

To date, there has been a concerted effort to better understand the host's immune response to infection with facultative intracellular bacterial pathogens such as *L. pneumophila*. However, in contrast to data available for cytokine responses to infection with *M. tuberculosis*, *L. monocytogenes* and other intracellular pathogens, the nature of the host immunologic response to *L. pneumophila* in terms of cytokine expression remains rudimentary. *In vitro* studies have focused on the importance of phagocytic cells parasitized by *L. pneumophila* and these have afforded much of our data on the intracellular replication of *L. pneumophila* (153, 250, 188, 370). Several studies have suggested the importance of selected cytokines including IFN-γ and TNF-α as tools for suppression of intracellular replication of *L. pneumophila* in both monocytes and macrophages *in vitro* (11, 31, 65, 149, 23, 249, 313).

The macrophage-like U-937 cell represents a useful model for investigating cellular infection by *L. pneumophila* as has been shown in this

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report and by others (153, 190, 267, 286). In the present study, intracellular replication of *L. pneumophila* in U-937 cells was shown to induce high levels of the proinflammatory cytokine IL-1β. Historically, the production of IL-1β and TNF-α by cells represents one of the first activation signals used by the host to combat infection. Interestingly, conflicting data exist regarding TNF-α production from cells *in vitro*. Matsiota *et al.* (230) showed that TNF-α levels in culture supernatant fluids from *L. pneumophila* infected human monocytes were low and were enhanced only after the addition of IFN-γ. However, Blanchard *et al.* (38) demonstrated that *Legionella*-infected monocytes were found to produce TNF-α in a dose-dependent response to the number of infecting bacteria, and the addition of recombinant IFN-γ to infected monocytes resulted in augmented production of TNF-α in a synergistic manner. In this study TNF-α was not elicited following *L. pneumophila* challenge *in vitro*. Furthermore, the viability of *L. pneumophila* appears to be relevant to the host response during infection, as U-937 cells cultured with heat-killed or formalin-fixed *L. pneumophila* failed to elicit a potent IL-1β response suggesting that viable bacteria in some way actively modify or induce the production of some cytokines. It is unlikely that heat killing of *L. pneumophila* alone completely destroyed important surface epitopes on this organism since challenge of U-937 cells with

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formalin- fixed *L. pneumophila* resulted in similar cytokine profiles.

Macrophages readily support intracellular replication of *L. pneumophila* while polymorphonuclear leukocytes possess potent bacteriocidal activities for this organism (86, 186). Based on this observation the ability of *L. pneumophila* to modify the production of IL-8, a potent chemo-attractant and activator of neutrophils, by macrophages was examined. It was demonstrated in this study that IL-8 is secreted from *Legionella*-infected cells. However, U-937 cells cultured with heat-killed *Legionella* produced significantly greater levels of IL-8 as compared to live organism challenge, at all time points investigated. Heat killing of *L. pneumophila* may result in the unmasking of cryptic epitopes that may be responsible for this observed effect. In addition, formalin fixation which maintains the structure of the outer surface of bacteria, indicates that the structure responsible for the increased levels of IL-8 may be located on the surface of the organism. Further studies were conducted to delineate whether the molecule(s) responsible for the increased levels of IL-8 following heat killing of *L. pneumophila* were located on the surface of the organisms or were being secreted by *L. pneumophila*. These studies included challenging U-937 cells for their ability to induce cytokines with either the bacterial pellet or the wash fluids from *L. pneumophila* following heat inactivation. These results indicated that the pellet was able to induce significantly greater levels of IL-8 as compared to the supernatant fluid. However, the supernatant fluid alone also resulted in a

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greater level of IL-8 produced from U-937 cells as compared to live organism challenge. Thus, preliminary data suggests that epitopes on the surface of the organism when heat-killed are in part responsible for the increased levels of IL-8 secreted from U-937 cells. That the supernatant wash fluid from heat-killed organisms resulted in a significantly greater level of IL-8 than did live *L. pneumophila* suggested that the mechanism of IL-8 induction is a multifactorial process and both surface and secreted molecules may by responsible for the increased IL-8 response. That the molecule responsible for this cytokine induction is the MOMP of *Legionella* cannot be excluded. The significant difference in levels of IL-8 secreted from U-937 cells treated with heat-killed *L. pneumophila* versus live *L. pneumophila* is interesting in that this chemokine is involved in neutrophil activation and recruitment *in vivo*. This may represent one mechanism by which *Legionella* limits neutrophil accumulation at the site of infection. In this fashion the local chemokine production is interrupted, thus allowing subsequent invasion of alveolar macrophages to precede. The importance of this observation cannot be overstated and requires further investigation in order to elucidate the importance of IL-8 and other chemokines in the outcome of Legionnaires' disease.

Park *et al.* (264) showed that peripheral blood monocytes challenged *in vitro* with *L. pneumophila* produce IL-10. In contrast, in this study, infection of U-937 cells with *L. pneumophila* resulted in a down-regulation of IL-10 as

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compared to uninfected controls. In these studies, IL-10 was constitutively expressed from phorbol ester treated U-937 cells. Challenge of U-937 cells with viable *L. pneumophila* suppressed IL-10 levels in culture supernatant fluids even below those levels expressed by uninfected cells. Interestingly, U-937 cells challenged with either heat-killed or formalin-fixed *L. pneumophila* produced significantly greater levels of IL-10 than did cells infected with live *L. pneumophila* or control cultures indicating that viable *L. pneumophila* suppresses IL-10 production. To assess where the cytokine inducing molecules were located on *L. pneumophila*, similar studies to those conducted for IL-8 were performed for IL-10 and similar results were obtained. Indeed, like IL-8, the results for IL-10 indicated that the molecule(s) responsible for the increased level of this cytokine were located on the bacterial surface; however, this molecule(s) may also be secreted from the bacterial surface. Again, the MOMP of *Legionella* is a likely candidate molecule. Work by Blander and Horwitz (43, 42, 41) have shown that the metalloprotease and outer membrane material of *L. pneumophila*, elicit cellular immune responses in guinea pigs. It is known that IL-10 is a potent down-regulator of host immune response (118, 147, 148, 199, 309). Previous studies have addressed the importance of IL-10 during *L. pneumophila* infection of cells by administering exogenous IL-10 to human alveolar macrophages and monocytes and suggest that treatment of alveolar macrophages with IL-10 exacerbated *Legionella* infection (264). Thus, based

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on these results it was expected that IL-10 would be produced from U-937 cells following challenge with a permissive infection. Interestingly, this was not what was observed. This would suggest that the interaction of *L. pneumophila* with U-937 cells resulted in decreased levels of IL-10 and this led to a permissive cellular infection.

It is widely accepted that a potent host immune response is required for resolution of Legionnaires' disease. Therefore, it was expected that cells infected with *L. pneumophila* would produce high levels of those cytokines well recognized to be important mediators of cell-mediated host immune response. However, the *in vitro* studies did not detect appreciable levels of IL-12 or IFN-γ in culture supernatant fluids. These results may offer one reason why U-937 cells infected with *L. pneumophila* are readily destroyed. This may represent a limitation of *in vitro* studies that use a single cell type in monoculture. That exogenous IFN-γ treatment of monocytes resulted in down-regulation of host cell infection further supports the suggestion that the source of IFN-γ may not come from macrophages but from another cell type (31, 249). The importance of this observation for the establishment and progression of Legionnaires' disease requires further study to better understand which cells in the lungs produce IFN-γ during infection. In addition, the complex interactions of specific cytokines in response to *L. pneumophila* infection need to be addressed if a rationale is to be developed

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so that the host's response may be manipulated to combat disease. In this study, the host response to *L. pneumophila* was characterized by investigating several classes of cytokines produced during *L. pneumophila* challenge.

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CHAPTER V

IN VIVO ANIMAL MODEL OF EXPERIMENTAL LEGIONNAIRES' DISEASE

5.1 Abstract

An experimental model of Legionnaires' disease was established in the A/J mouse using *Legionella pneumophila* strain Nottingham 7. Initially, an effective dose $_{50}$ (ED_{50}) was established with this strain and mouse lungs were harvested for both gross pathology and histopathology. An ED_{50} was defined as the inoculum of *L. pneumohila* that resulted in 50 % of the infected mice becoming mouribund at which point it was determined that they would not recover from infection and were euthanized. Following the ED_{50} study, a sublethal infection of *L. pneumophila* was used to investigate the replication of bacteria and the pathology of the resultant pneumonic changes induced in the lungs of mice over time. The ED_{50} was calculated to be $4.63x10^6$. Pathological and histopathological changes were evident when challenges of 10^6 to 10^9 organisms were used. To better characterize the immunostimulatory effect of *L. pneumophila in vivo*, A/J mice were infected intratracheally with a sublethal dose ($1x10^6$ CFU) of either live or heat-killed *L. pneumophila*. Live challenge resulted in an initial 8-fold

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increase in the numbers of *L. pneumophila* in the lungs within an initial 24 h period and this was followed with gradual clearing of the organism by 120 h. Gross pathologic and histopathologic evaluations revealed a progression of diseased state. Bronchoalveolar lavage (BAL) fluids, lung homogenates and sera were collected from mice challenged with live or heat-killed *L. pneumophila* and were tested for the presence of various cytokines in response to *Legionella* infection. BAL fluids and homogenates of lung tissue from *L. pneumophila*-infected mice contained significantly higher levels of IL-1β, TNF-α, macrophage inflammatory protein 2 (MIP-2), IL-12 (p40 and p70), and IFN-γ as compared to uninfected animals. Serum cytokine levels demonstrated a systemic rise in IL-1β, MIP-2, IL-12 (p40 and p70) and IFN-γ from *L. pneumophila*-infected A/J mice. TNF-α was not detected in the sera of A/J mice challenged with *L. pneumophila*. IL-10 was not detected in any samples from either infected or control mice. A/J mice challenged with heat-killed *L. pneumophila* did not develop gross pathologic or histopathologic changes and failed to elicit significant levels of cytokines in BAL fluids, lung homogenates or sera. These results suggest that *L. pneumophila* elicited a potent yet specific cytokine response in A/J mice.

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5.2 Introduction

The use of single cell systems is critical to understanding how individual cell types respond to challenge with *L. pneumophila.* However, these systems provide limited insight into the complex interactions involved in the infection of an animal host. Historically, the guinea pig (132, 120, 87), rat (315) and hamster (263) models have been used to study the progression of Legionnaires' disease, but have not been useful for studying the host immune response to *L. pneumophila* infection, in part, due to the lack of specific immunologic reagents available for these species. The use of animal models, primarily the guinea pig, have yielded insight into the underlying processes that occur following inhalation of *L. pneumophila* into the lung and have confirmed the host cellular response to *L. pneumophila* challenge. Yamamoto *et al.* (370) established that peritoneal macrophages of A/J mice supported *L. pneumophila* replication, while peritoneal macrophages from other mouse strains, including BALB/c, BDF1, C3H/HeN, C57BL/6 and DBA/2, were resistant to *Legionella* infection. Prior to these studies it was believed that mice were uniformly resistant to *L. pneumophila* infection and thus represented a poor model for studying this condition.

Recently, there has been a recrudescence in the interest to better understand the host's immune response, particularly to elucidate any role that cytokines may play in regulating infection systemically. To date there is limited information regarding the production of cytokines during *L.*

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pneumophila infection in animal models. Brieland *et al.* (55) developed a surgical model of Legionnaires' disease pneumonia using A/J mice and this has been shown to closely mimic the progression of human disease in many respects. A major advantage of this development is the abundance of immunologic reagents available for the mouse and this has allowed for a more intense study of the immune response following pneumonic infection with *Legionella*. IFN-γ, TNF-α and IL-12, as well as nitric oxide, have been detected in lung homogenate fluids of A/J mice challenged with *L. pneumophila* (55, 58, 59). Recently, Brieland *et al.* (59) showed that mice depleted of IL-12 by treatment with an IL-12 specific blocking monoclonal antibody, developed an increase in *L. pneumophila* replication with exacerbated pneumonic changes in the lungs of mice following challenge. Although not characterized definitively, it was suggested by these workers, that inhibition of IL-12 was presumably functioning through the modulation of TNF-α.

To develop a better understanding of the complex immunologic response to *L. pneumophila, in vivo* studies were conducted using A/J mice, to study experimental Legionnaires' disease. Both gross pathology and histopathology were documented during infection with live or inoculation with heat-killed *L. pneumophila*. In addition, the production of several classes of cytokines in response to *L. pneumophila* infection were investigated.

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5.3 Materials and Methods

Reagent formulations and preparation along with detailed procedures are given in Appendix 2.

5.3.1 Bacterial Cultivation

L. pneumophila serogroup 1, strain Nottingham N7 was grown and maintained as previously described (see Section 2.3.1). Following 24 h of growth in 5 ml of BYE-α media, 1 ml of *L. pneumophila* was placed in sterile Eppendorf tubes and centrifuged at 6,000 x g for 5 min. Supernatant fluid was removed and the bacterial pellet was washed three times with pyrogen-free Dulbecco's phosphate buffered saline (DPBS) (Cellgro). Bacteria were then resuspended in 1 ml fresh DPBS and diluted to give a series of inocula of $1x10^5$ to $1x10^9$ CFU for animal effective dose $_{50}$ (ED_{50}) studies. Subsequent sublethal challenge experiments used inocula of $1x10^6$ CFU. For heat-kill studies, *L. pneumophila* ($1x10^6$ CFU) in DPBS was boiled for 10 min. Heat killing was confirmed by plating organisms on BCYE-α. In addition, all bacterial inocula used for ED_{50} and sublethal experiments were serially diluted and plated on BCYE-α to determine CFU/ml of *L. pneumophila*.

5.3.2 Intratracheal Inoculation of Mice

A colony of A/J mice was established at the University of New

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Hampshire animal facility by breeding siblings from the same liter (Jackson Laboratories, Bar Harbor, Maine). Mice received food and water ad libitum and were housed, cared for and bred in accordance with NIH and University of New Hampshire Animal Care and Use Committee (ACUC) guidelines under protocol # 960905. Male or female inbred A/J mice 6 to 8 weeks old were placed into six groups each of 5 mice and anesthetized with 0.1 ml of a 10 mg/ml solution of ketamine and xyaline. The skin of the ventral neck was shaved, swabbed with a tincture of iodine and an incision made through the skin of the ventral neck and the trachea isolated. For ED_{50} studies, a 40 µl volume of various doses ($1x10^5$ to $1x10^9$ CFU) of *L. pneumophila* in pyrogen-free DPBS was injected into the trachea of different groups of mice using a 1 ml syringe with a 27-gauge needle (Plate 5.1). For sublethal challenge studies an inoculum of $1x10^6$ CFU of *L. pneumophila* was used for each mouse. The bacterial challenge inoculum was followed by 10 µl of air. Control animals received DPBS only. Three 4-0 absorbable subcuticular sutures were used to close the incision. Following challenge mice were placed in HEPA filter top cages and their behavior and eating patterns were observed six times daily over a period of 12 days. This included failure to groom, loss of mobility, failure to show normal patterns of activity and abnormal resting postures in which the animal appeared to be sleeping or was hunched up. Moribund mice were sacrificed by CO_2 asphyxiation. The ED_{50} value was determined by the method of Reed and Meunch.

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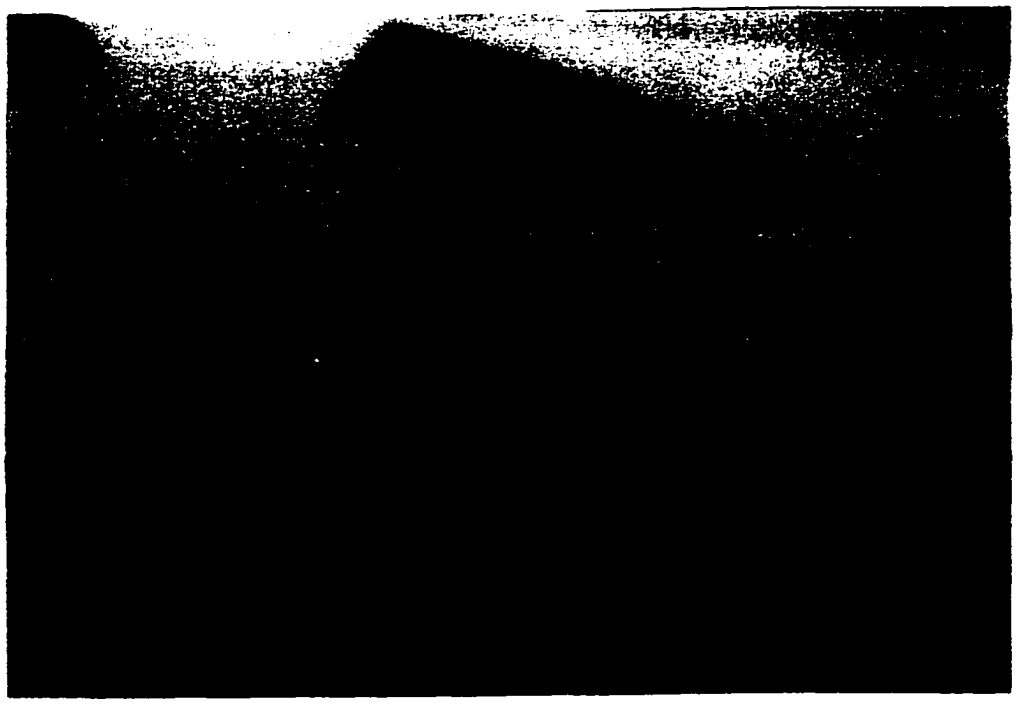

Plate 5.1. Intratracheal inoculation of *L. pneumophila* into an A/J mouse that has been anesthetized with ketamine and xyaline.

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5.3.3 Gross Pathology and Histopathology

Following challenge with either live or heat-killed *L. pneumophila*, mice were euthanized by CO_2 asphyxiation and lungs were removed and photographed for gross pathologic purposes. For histopathologic preparation, the trachea of euthanized mice was isolated, tied off and the lungs were perfused with 1 ml of a 10 % neutral buffered formalin solution. After 1 min, lungs were removed and stored in the same solution for processing by histologic procedures (Appendix 2). Briefly, lung samples were placed into histology embedding cassettes and washed overnight in water. Tissues were dehydrated in an ethanol series (70-100 %), placed in the clearing agent, Histoclear II (National diagnostics, Atlanta, GA.) and embedded in paraffin wax using a Shandon Citadel Tissue Processor (Shandon Lipshaw, Pittsburg, PA.). Six micron thick sections were cut using an American Optical Microtome and sections were deparaffinized, stained with Harris' Alum Hematoxylin and 0.1 % eosin (Appendix 2) and examined histopathologically using an Olympus optical microscope. Duplicate samples were stained with Steiner's stain (Appendix 2) for organism detection.

5.3.4 Replication of *L. pneumophila* in Lungs of A/J Mice and Detection of Cytokine Production

Time course studies were performed to characterize the replication of *L. pneumophila* N7 in the lungs of mice following sublethal challenge.

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Initially following surgery as well as at 12, 24, 48, 72, 96 and 120 h post-infection groups of animals (n = 5) were humanly euthanized by CO_2, according to ACUC protocol # 960905, and the lungs from challenged and control mice were aseptically harvested, weighed and homogenized for 5 min on ice in 10 ml sterile pyrogen-free DPBS in an electric Omni homogenizer. A 100 µl aliquot of each homogenate was serially diluted 10-fold and duplicate 25 µl samples of each dilution were plated on BCYE-α selective agar containing polymyxin B, anisomycin and cefamandole (BCYE + PAC, Becton-Dickinson, Cockeysville, MD). *Legionella* colonies were enumerated after 72 h growth and the concentration of *L. pneumophila* were expressed as CFU per gram of lung tissue. To determine the levels of cytokines elicited following *L. pneumophila* infection, A/J mice were challenged with either a sublethal dose of live *L. pneumophila* or an equivalent number of heat-killed *L. pneumophila* as determined by previous growth curve studies conducted for the organism. At similar time points, BAL fluids and serum samples were collected from A/J mice (n = 5 per group). To collect BAL fluids, the trachea of sacrificed animals was tied off and the lungs lavaged with 1 ml pyrogen-free DPBS using a syringe with a 27-gauge needle. All lavages yielded similar volumes, with approximately 90 % recovery. BAL fluids were centrifuged at 6,000 x g for 10 min, filtered through a 0.2 µm filter, transferred to sterile Eppendorf tubes and stored frozen at -70°C until assayed for cytokine levels using murine cytokine-specific ELISA kits (R & D systems, Minneapolis,

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Minn.). Blood was collected from the dorsal aorta using a 1 ml syringe with a 27-gauge needle, placed into sterile tubes, allowed to clot for 4 h at 4°C, then centrifuged at 1,000 x g for 10 min. Sera were collected, placed into sterile Eppendorf tubes and stored frozen at -70°C until assayed for levels of cytokines.

In separate experiments, lung homogenates were collected from similarly infected A/J mice. Lungs were harvested and homogenized and a 1 ml sample of lung homogenate from challenged (live or heat-killed *L. pneumophila*) or control mice was centrifuged at 6,000 x g for 10 min. The supernatant fluid was filtered, transferred to sterile Eppendorf tubes and stored frozen at -70°C until assayed for cytokines. The non-parametric Mann-Whitney test was performed as described in section 4.3.5 to analyze differences in cytokine levels from BAL fluids, lung homogenates and sera of A/J mice that received DPBS, viable or killed legionellae.

5.4 Results

5.4.1 Pathogenesis and pathology. The ED_{50} value for *L. pneumophila* strain Nottingham 7 infection of A/J mice was determined and is illustrated in Table 5.1. The ED_{50} was calculated mathematically by the Reed and Meunch method to be 4.63×10^6 CFU/mouse. Gross and histopathological lesions induced in A/J mice after inoculation intratracheally with *L. pneumophila* were evident in the lungs and distributed throughout both

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Table 5.1. Effective dose $_{50}$ study administering *L. pneumophila* N7 to A/J mice via intratracheal challenge

Challenge CFU *L. pneumophila*	Alive animals / Challenged animals
$1x10^5$	5 / 5
$1x10^6$	5 / 5
$1x10^7$	1 / 5
$1x10^8$	0 / 5
$1x10^9$	0 / 5

Five mice were inoculated with each dilution of *L. pneumophila* and the ED_{50} was calculated mathematically from duplicate experiments to be $4.63x10^6$ CFU. Control mice were injected with Dulbeccos phosphate buffered saline (DPBS) and in this group no deaths occured up to twelve days post-challenge.

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right and left lobes. Plate 5.2 depicts lungs harvested from A/J mice that had received $1x10^5$ to $1x10^9$ CFU of *L. pneumophila*. Control animals received DPBS. At $1x10^7$ CFU, A/J mouse lungs were pale in color indicative of necrosis, while at $1x10^8$ and $1x10^9$ CFU severe hemorrhaging was observed.

Histopathological examination revealed pathological changes in the lungs of infected mice receiving $1x10^5$ to $1x10^9$ CFU. At $1x10^5$ CFU the alveolar septa appeared thickened as compared to control sections, with the presence of congestion and possibly hemorrhage within the septa (Plate 5.3). Lung sections from animals that had received $1x10^6$ CFU exhibited an increase in cellular infiltrate and this consisted of PMNLs and mononuclear cells as detected at higher magnifications (Plate 5.4). In addition, edema was present and appeared as lightly pink staining areas while congestion of blood vessels was evident. At $1x10^7$ CFU, the perivascular space was thickened and showed an increased presence of red blood cells when compared with controls (Plate 5.4). Dilation of capillaries and hemorrhaging within the alveolar septa were notable and indicated lysis of capillaries. Both cellular and protein-rich exudate were evident in addition to a reduction in the number of alveoli as compared to control lungs. As shown in Plate 5.5, at $1x10^8$ CFU, hemorrhage into the alveoli occurred as a result of disruption of blood vessel walls and this resulted in an overall loss of lung architecture. Animals receiving $1x10^9$ CFU presented with severe congestion of blood vessels and hemorrhaging

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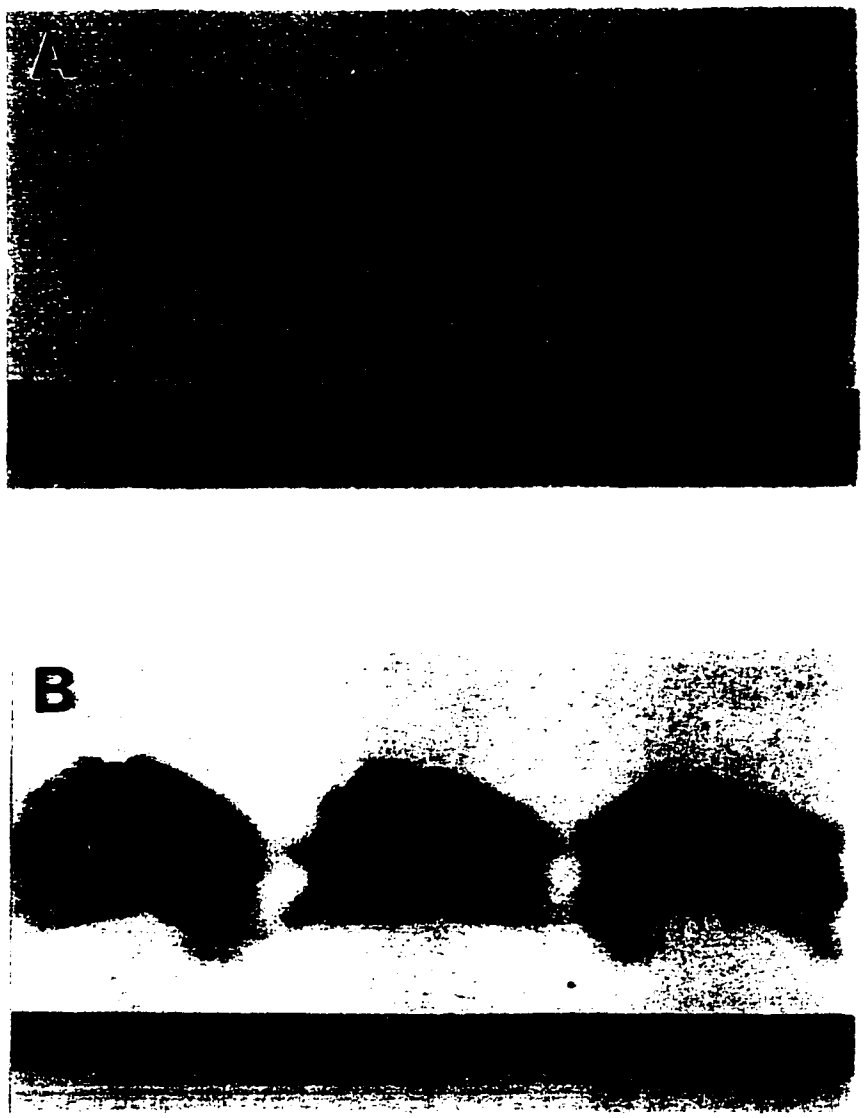

Plate 5.2. Lung gross pathology of A/J mice inoculated intratracheally with *L. pneumophila* during ED_{50} study.
A) From left to right; control lung from A/J mouse inoculated intratracheally with Dulbeccos phosphate buffered saline (DPBS), A/J mouse lungs infected with 10^5 and 10^6 CFU of *L. pneumophila* respectively. B) From left to right; A/J mouse lungs infected with 10^7, 10^8, and 10^9 CFU of *L. pneumophila*. At 10^7 CFU, A/J mouse lung is pale indicative of necrosis and at 10^8 and 10^9 CFU hemorrhaging is observed.

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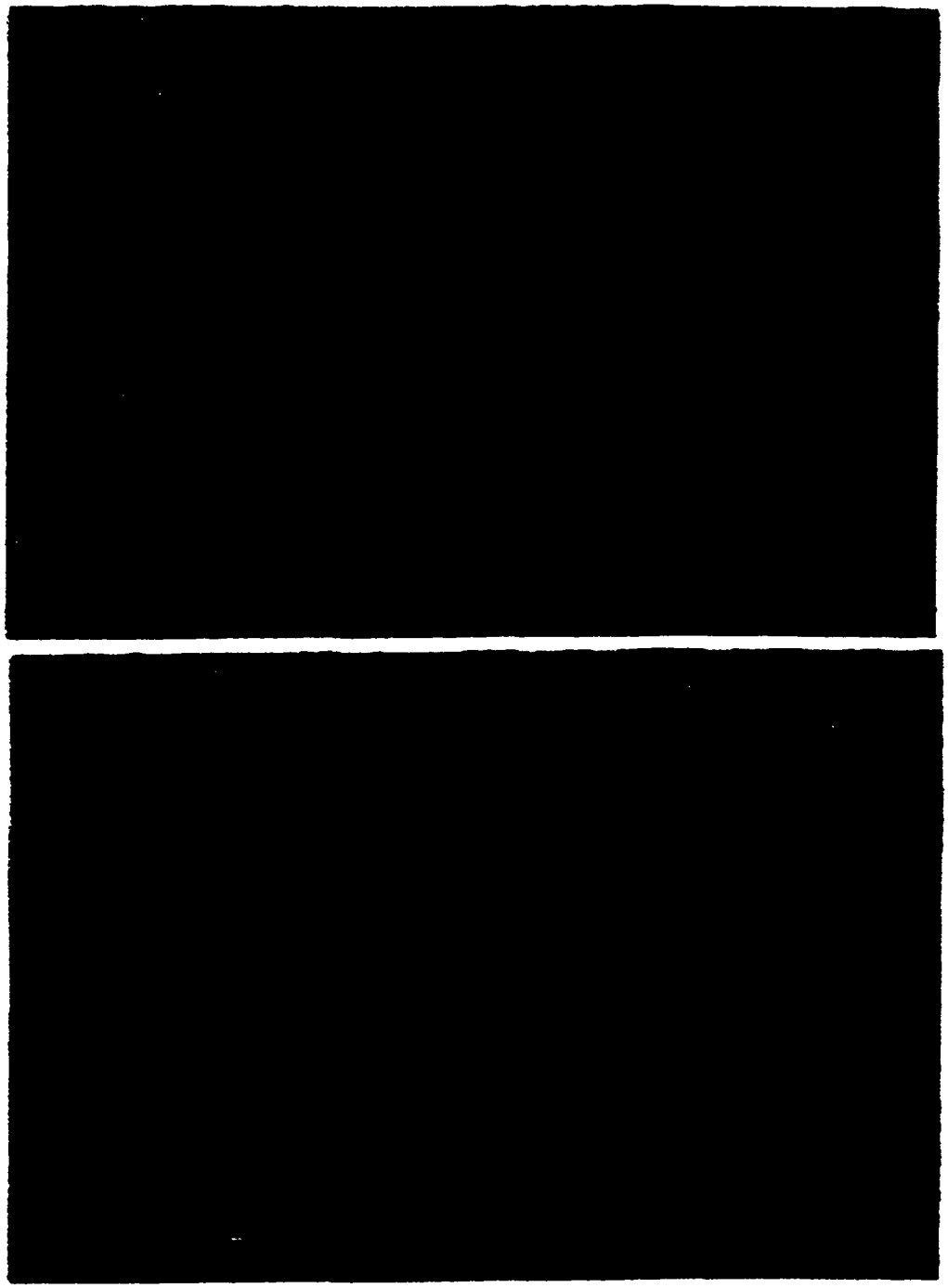

Plate 5.3. Histological sections of lung tissue from A/J mice inoculated intratracheally with *L. pneumophila* during ED_{50} study. Hematoxylin and eosin stain.
A) Control lung inoculated intratracheally with DPBS (X 255). B) A/J mouse lung infected with 10^5 CFU of *L. pneumophila*. Note thickened alveolar septa with increased presence of red blood cells (RBCs) as compared to control lungs (X 255).

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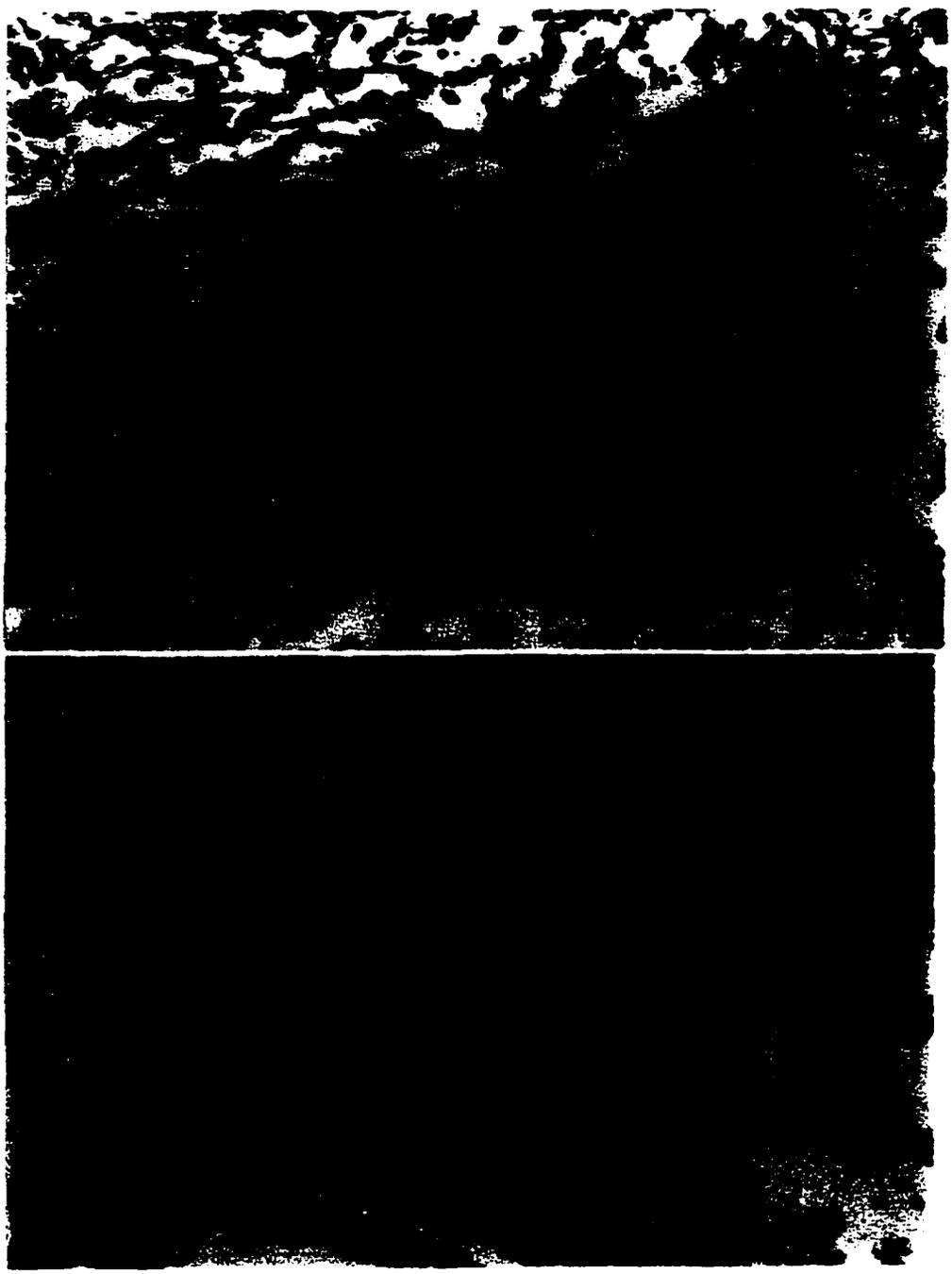

Plate 5.4. Histological sections of lung tissue from A/J mice inoculated intratracheally with *L. pneumophila* during ED_{50} study. Hematoxylin and eosin stain.
A) A/J mouse lung infected with 10^6 CFU of *L. pneumophila*. Note increase in cellular infiltrate (X 255). B) A/J mouse lung infected with 10^7 CFU of *L. pneumophila*. Note congestion of blood vessels, thickened alveolar septa, fibrin strands present as light pink staining areas and the presence of both cellular and protein-rich exudate (X 294).

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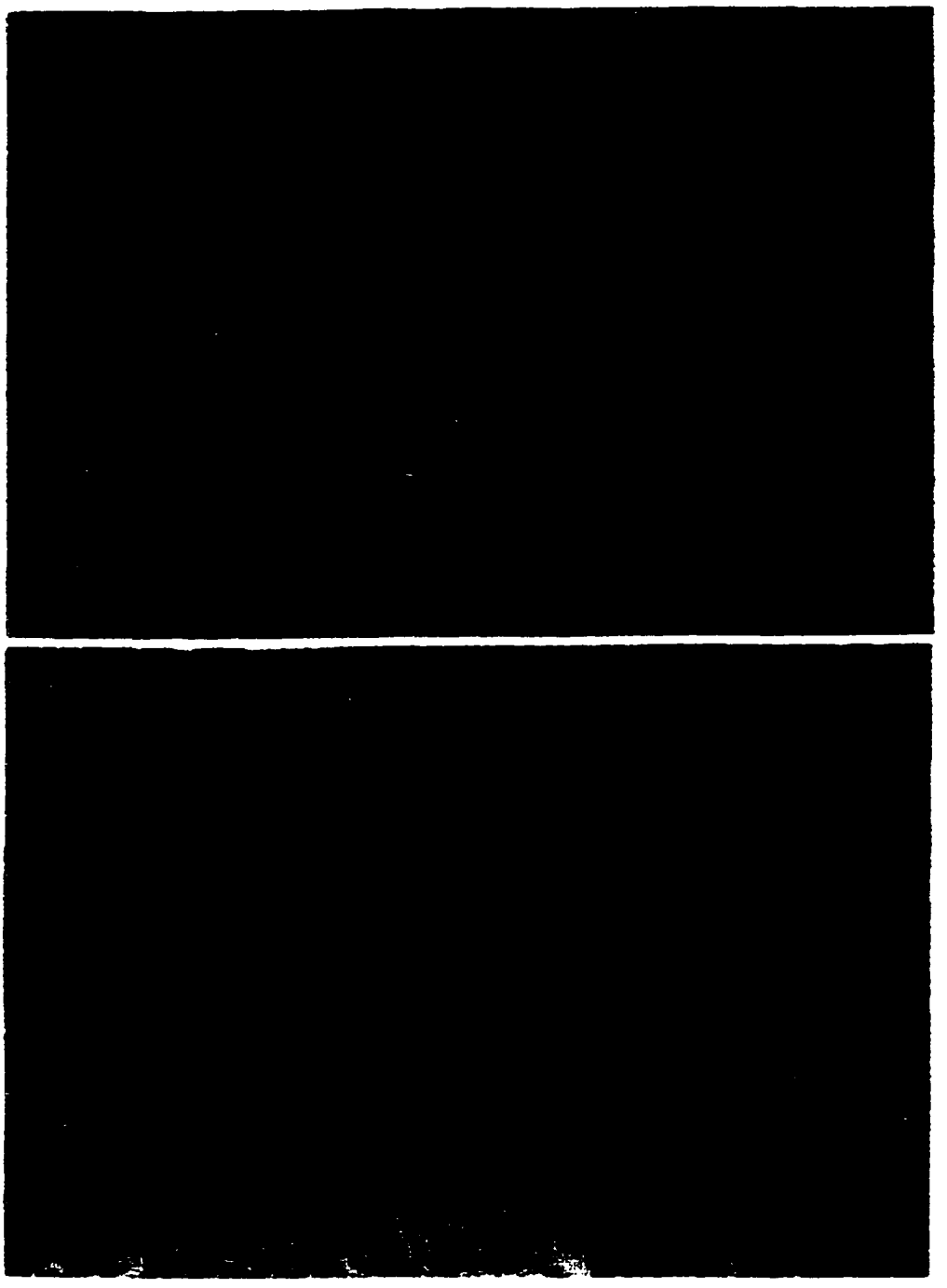

Plate 5.5. Histological sections of lung tissue from A/J mice inoculated intratracheally with *L. pneumophila* during ED_{50} study. Hematoxylin and eosin stain.
A) A/J mouse lung infected with 10^8 CFU of *L. pneumophila.* Note hemorrhage into alveoli (X 255). B) A/J mouse lung infected with 10^9 CFU of *L. pneumophila.* Note congestion of blood vessels and hemorrhaging, and bronchioles filled with protein-rich material (X 128).

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Plate 5.6. Histological sections of lung tissue from A/J mice inoculated intratracheally with 10^8 CFU of *L. pneumophila* during ED_{50} study. Steiner's stain. Note organisms within lung alveoli (X 1277).

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(Plate 5.5). In addition, bronchioles were filled with protein-rich material, indicative of a dysfunctional lung. As shown in Plate 5.6, *L. pneumophila* was visulalized during the ED_{50} study by using the silver nitrate based Steiner's stain.

5.4.2 **Sublethal infection.** Following the ED_{50} study, a sublethal dose ($1x10^6$ CFU) was chosen to investigate the *in vivo* replication of *L. pneumophila* with time. In addition, gross pathology and histopathology coinciding with bacteriologic replication were evaluated. *L. pneumophila* strain Nottingham 7 was able to replicate within the lungs of A/J mice and this resulted in both gross and histopathological changes. There was an 8-fold increase in *L. pneumophila* replication 12 to 24 h after intratracheal inoculation (Figure 5.1). Over the next 12 h (between 24 to 36 h), growth of *L. pneumophila* in the lung plateaued and at 48 to 120 h *L. pneumophila* was gradually cleared from the lungs, although low numbers of bacteria were still detected in lung homogenates of mice five days post infection.

During the course of *L. pneumophila* infection gross and histopathological evidence of infection were demonstrated in mice inoculated with $1x10^6$ CFU. At 24 h post-infection, changes in lung appearance were evident as compared to controls (Plate 5.7). Changes were observed during the course of infection and at 72 h and 96 h post-challenge,

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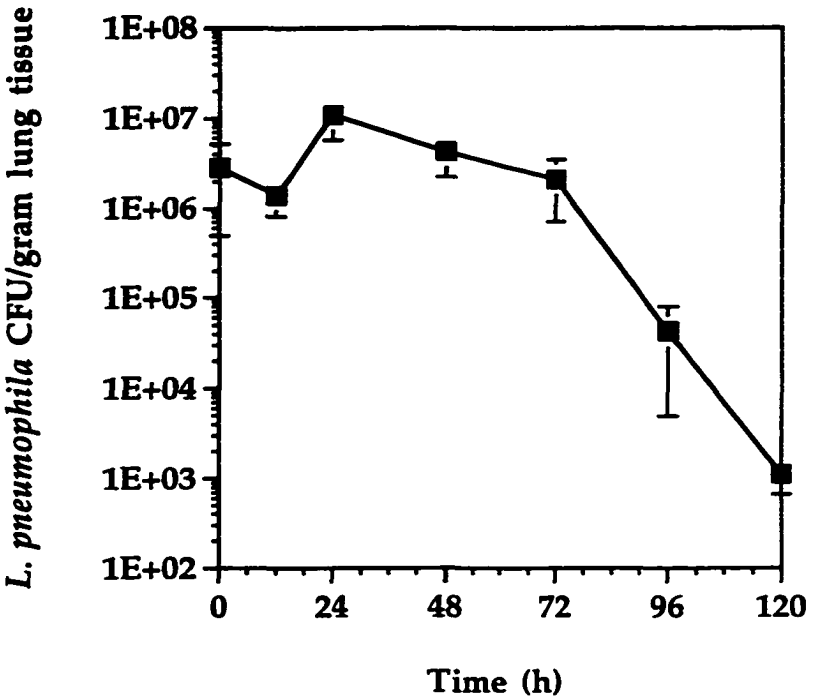

Figure 5.1: Replication of *L. pneumophila* in the lungs of A/J mice over time. *L. pneumophila* was administered ($1x10^6$ bacteria per mouse) to A/J mice intratracheally, animals were euthanised at the indicated time points and lungs were removed and homogenized. Tissue homogenates were diluted and plated onto BCYE-PAC plates and colonies were counted after 72 h. Results are expressed as means ± standard deviations from five separate experiments each using five mice per time point. Data are calculated as CFU/ gram lung tissue.

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hemorrhagic necrosis was present in the right and left lobes of the lung (Plate 5.8 and 5.9). At 120 h post-infection, the lobes of the lung were often dark in color due to hemorrhage and the lungs appeared pale as compared to controls which were pink and healthy looking (Plate 5.9). Histopathological changes were documented over time during the course of *L. pneumophila* infection. Plates 5.10 through 5.17 illustrate the progression of infection during replication of *L. pneumophila* within A/J mouse lungs. Histopathological changes were evident by 24 h post-infection (Plate 5.12) and were present throughout the course of the infection including at 120 h post-challenge (Plate 5.17), when, according to VBCC counts (Figure 5.1), only low number of *L. pneumophila* were present. These changes included increased cellular and proteinaceous exudate, extreme thickening of the interalveolar septa, a reduction in the number and structure of opened alveoli and an increased presence of red blood cells as compared to control sections (Plate 5.11-5.15). Throughout the course of infection alveolar wall thickening was observed and fibrin deposits in the alveoli were present by 72 h post-infection (Plate 5.15). Bronchi and bronchioles were essentially normal in appearance, except at later time points (Plate 5.16). Edema with scattered PMNLs were noted in perivascular spaces during infection (Plates 5.15 and 5.16). In addition, to characterize the effect of *L. pneumophila* viability on the organism's ability to induce pathologic and histopathologic changes *in vivo*, A/J mice were challenged intratracheally with a sublethal dose ($1x10^6$ CFU) of heat-killed *L.*

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Plate 5.7. Lung gross pathology of A/J mice inoculated intratracheally with a sublethal dose (10^6 CFU) of *L. pneumophila.* A/J mice were challenged with either live or heat-killed *L. pneumophila.*
A) Control lung from A/J mouse inoculated intratracheally with DPBS. B) A/J mouse lung challenged with heat-killed *L. pneumophila* 6 h post-challenge. C) A/J mouse lung infected with live *L. pneumophila* 6 h post-challenge. D) A/J mouse lung challenged with heat-killed *L. pneumophila* 24 h post-challenge. E) A/J mouse lung infected with live *L. pneumophila* 24 h post-challenge.

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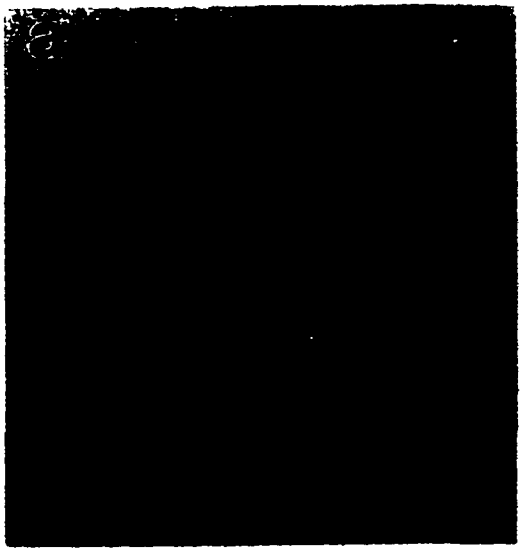

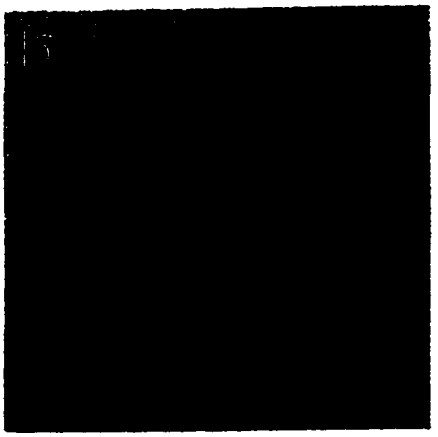

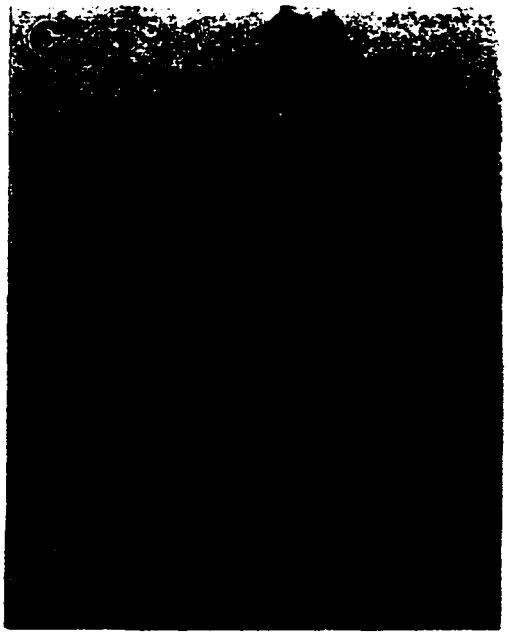

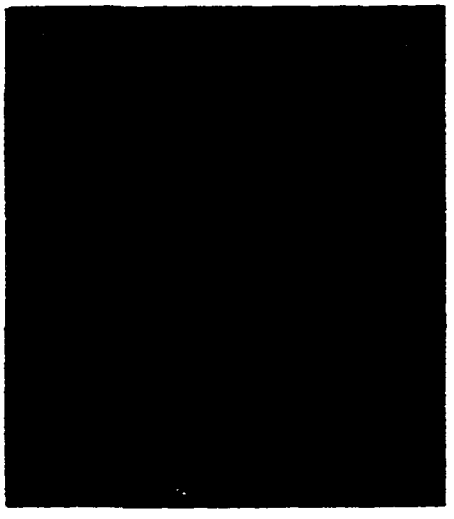

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Plate 5.8. Lung gross pathology of A/J mice inoculated intratracheally with a sublethal dose (10^6 CFU) of *L. pneumophila*. A/J mice were challenged with either live or heat-killed *L. pneumophila*.
A) A/J mouse lung challenged with heat-killed *L. pneumophila* 48 h post-challenge. B) A/J mouse lung infected with live *L. pneumophila* 48 h post-challenge. C) A/J mouse lung challenged with heat-killed *L. pneumophila* 72 h post-challenge. D) A/J mouse lung infected with live *L. pneumophila* 72 h post-challenge. Note hemorrhagic necrosis in upper left and lower right lobes of lung.

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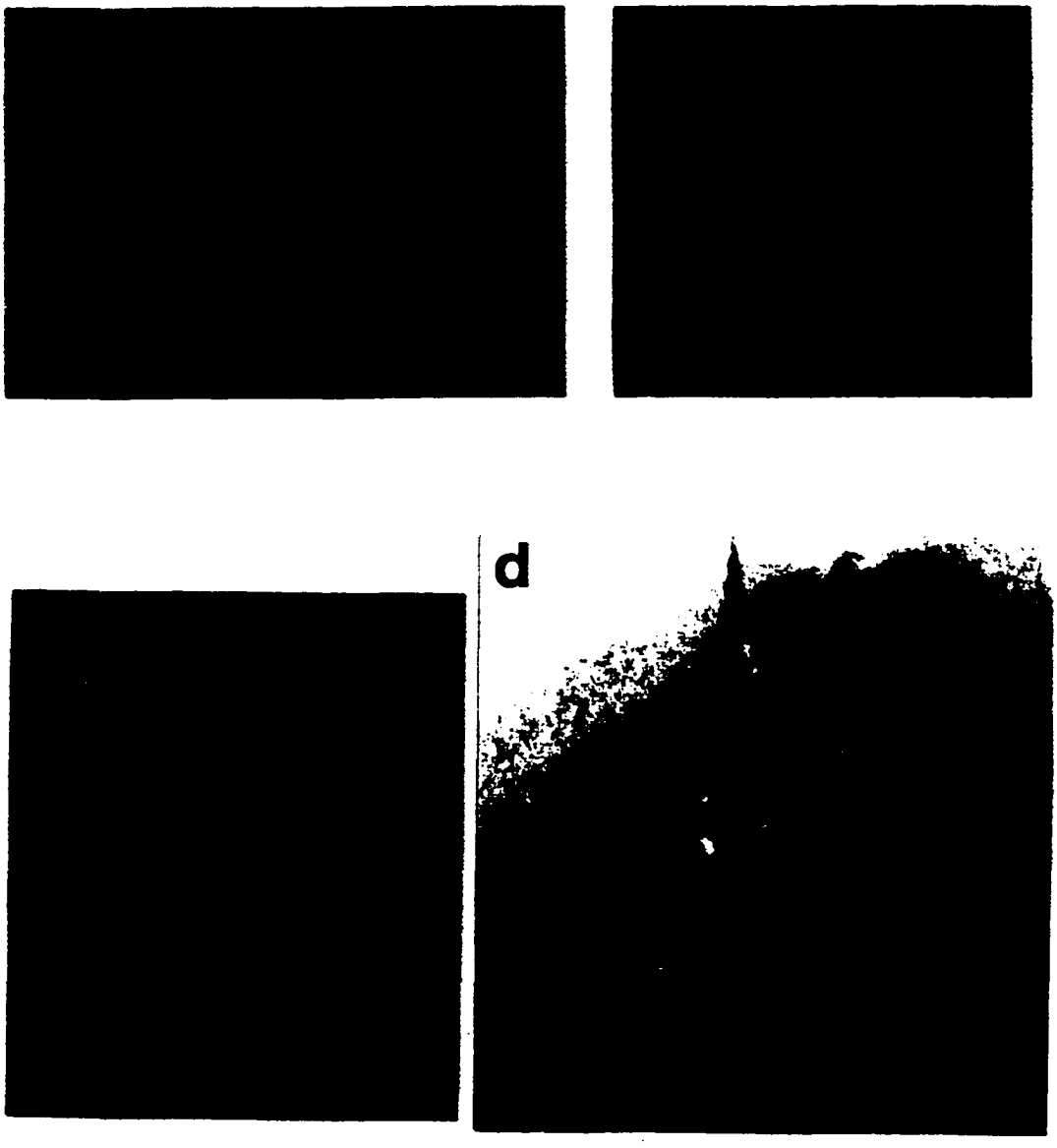

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Plate 5.9. Lung gross pathology of A/J mice inoculated intratracheally with a sublethal dose (10^6 CFU) of *L. pneumophila.* A/J mice were challenged with either live or heat-killed *L. pneumophila.*
A) A/J mouse lung challenged with heat-killed *L. pneumophila* 96 h post-challenge. B) A/J mouse lung infected with live *L. pneumophila* 96 h post-challenge. Note hemorrhagic necrosis in both lobes of lung. C) A/J mouse lung challenged with heat-killed *L. pneumophila* 120h post-challenge. D) A/J mouse lung infected with live *L. pneumophila* 120 h post-challenge. Note hemorragic necrosis in upper left lobe of lung.

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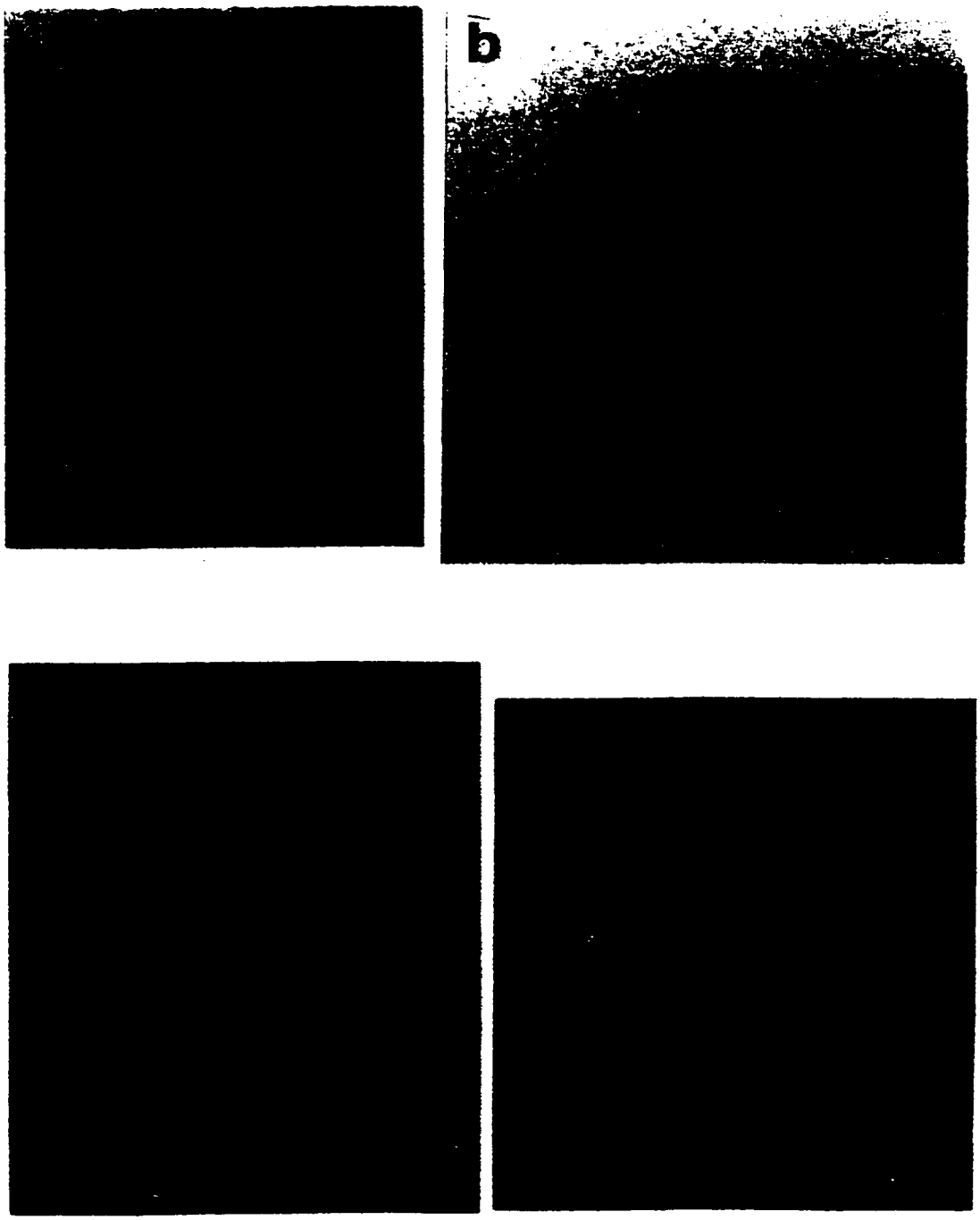

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Plate 5.10. Histological sections of lung tissue from A/J mice inoculated intratracheally with a sublethal dose (10^6 CFU) of *L. pneumophila* 6 h post-challenge. A/J mice were challenged with either live or heat-killed *L. pneumophila*. Hematoxylin and eosin stain.
A) Control lung from A/J mouse inoculated intratracheally with DPBS (X 255). B) A/J mouse lung challenged with heat-killed *L. pneumophila* (X 255). C) A/J mouse lung infected with live *L. pneumophila* (X 255).

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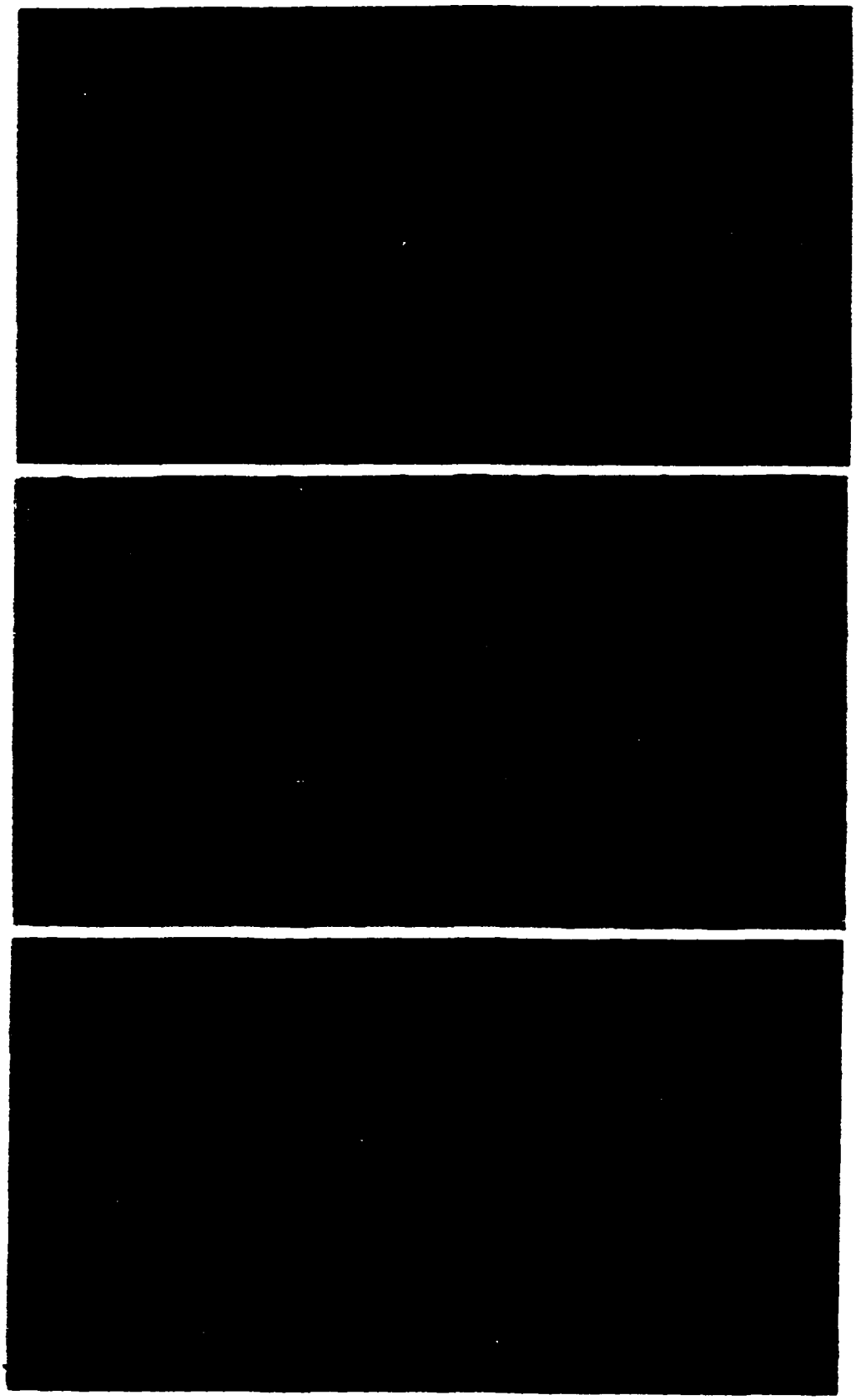

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Plate 5.11. Histological sections of lung tissue from A/J mice inoculated intratracheally with a sublethal dose (10^6 CFU) of *L. pneumophila* 12 h post-challenge. A/J mice were challenged with either live or heat killed *L. pneumophila*. Hematoxylin and eosin stain.
A) Control lung from A/J mouse inoculated intratracheally with DPBS (X 255). B) A/J mouse lung challenged with heat-killed *L. pneumophila* (X 255). C) A/J mouse lung infected with live *L. pneumophila*. Note RBCs and few inflammatory cells within blood vessel (X 255).

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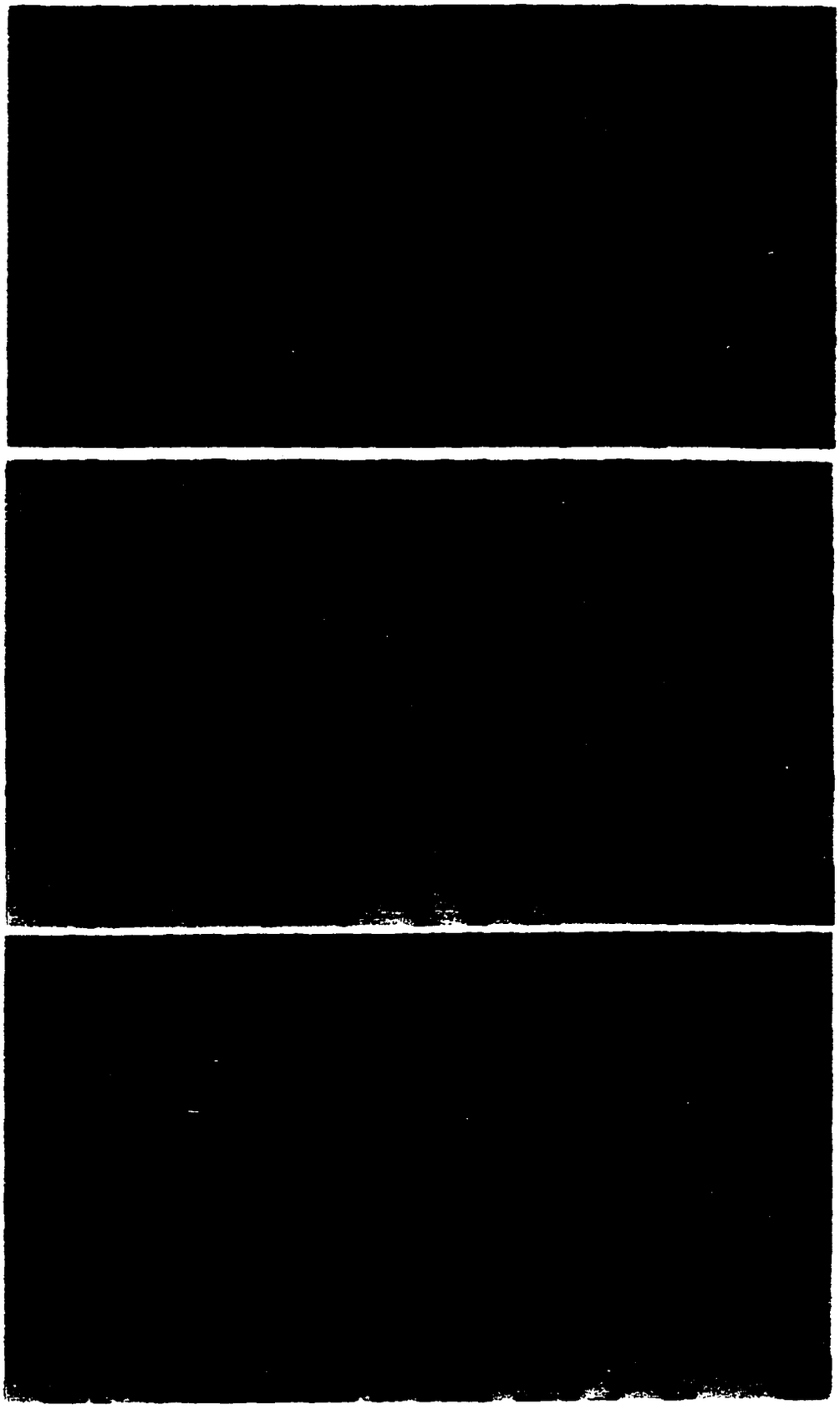

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Plate 5.12. Histological sections of lung tissue from A/J mice inoculated intratracheally with a sublethal dose (10^6 CFU) of *L. pneumophila* 24 h post-challenge. A/J mice were challenged with either live or heat-killed *L. pneumophila*. Hematoxylin and eosin stain.
A) Control lung from A/J mouse inoculated intratracheally with DPBS (X 255). B) A/J mouse lung challenged with heat-killed *L. pneumophila* (X 255). C) A/J mouse lung infected with live *L. pneumophila*. Note thickened interalveolar septa and increase of inflammatory cells (X 255).

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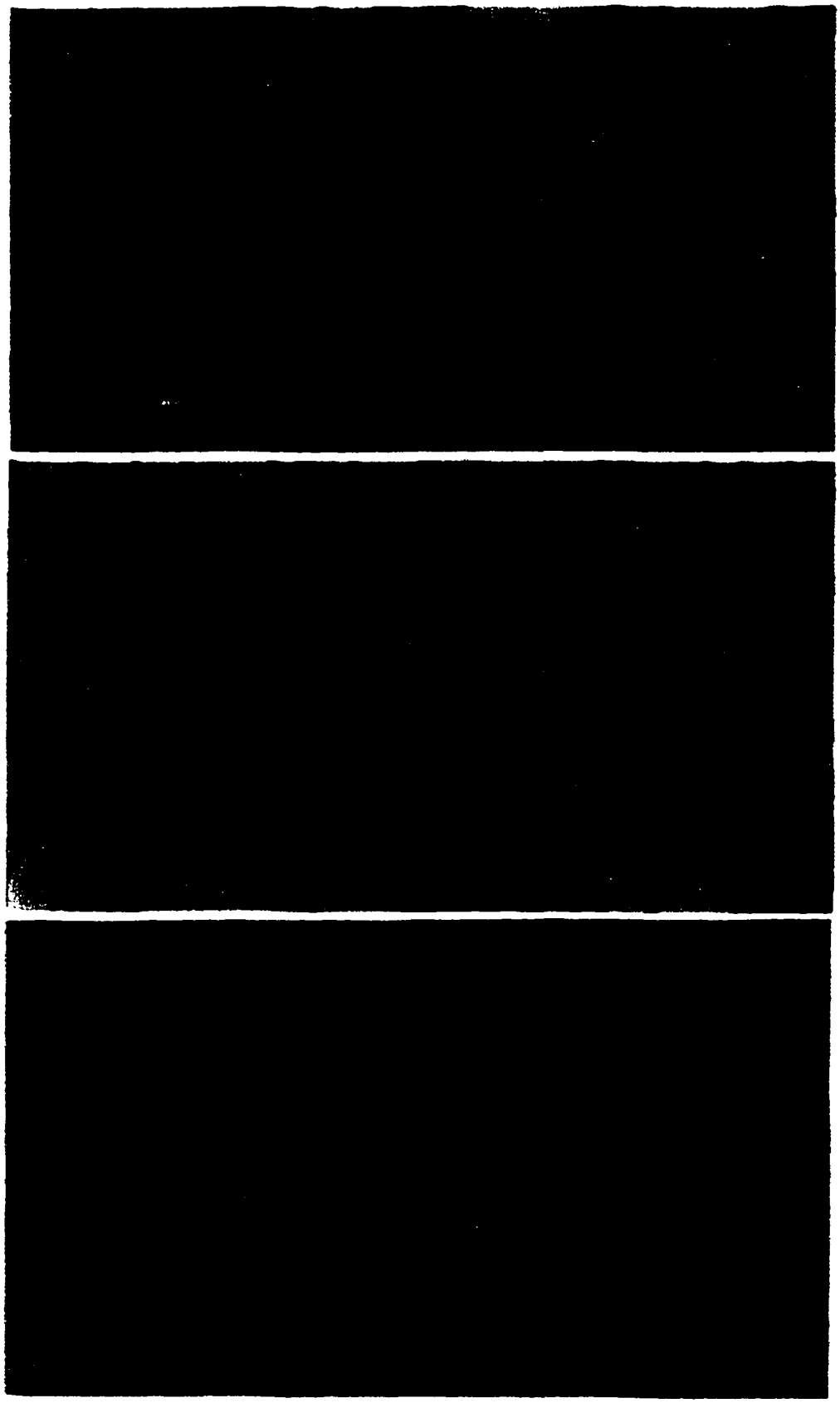

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Plate 5.13. Histological sections of lung tissue from A/J mice inoculated intratracheally with a sublethal dose (10^6 CFU) of *L. pneumophila* 36 h post-challenge. A/J mice were challenged with either live or heat killed *L. pneumophila.* Hematoxylin and eosin stain.
A) Control lung from A/J mouse inoculated intratracheally with DPBS (X 255). B) A/J mouse lung challenged with heat-killed *L. pneumophila* (X 255). C) A/J mouse lung infected with live *L. pneumophila.* Note thickened interalveolar septa and increase of inflammatory cells and limited number of opened alveoli (X 255).

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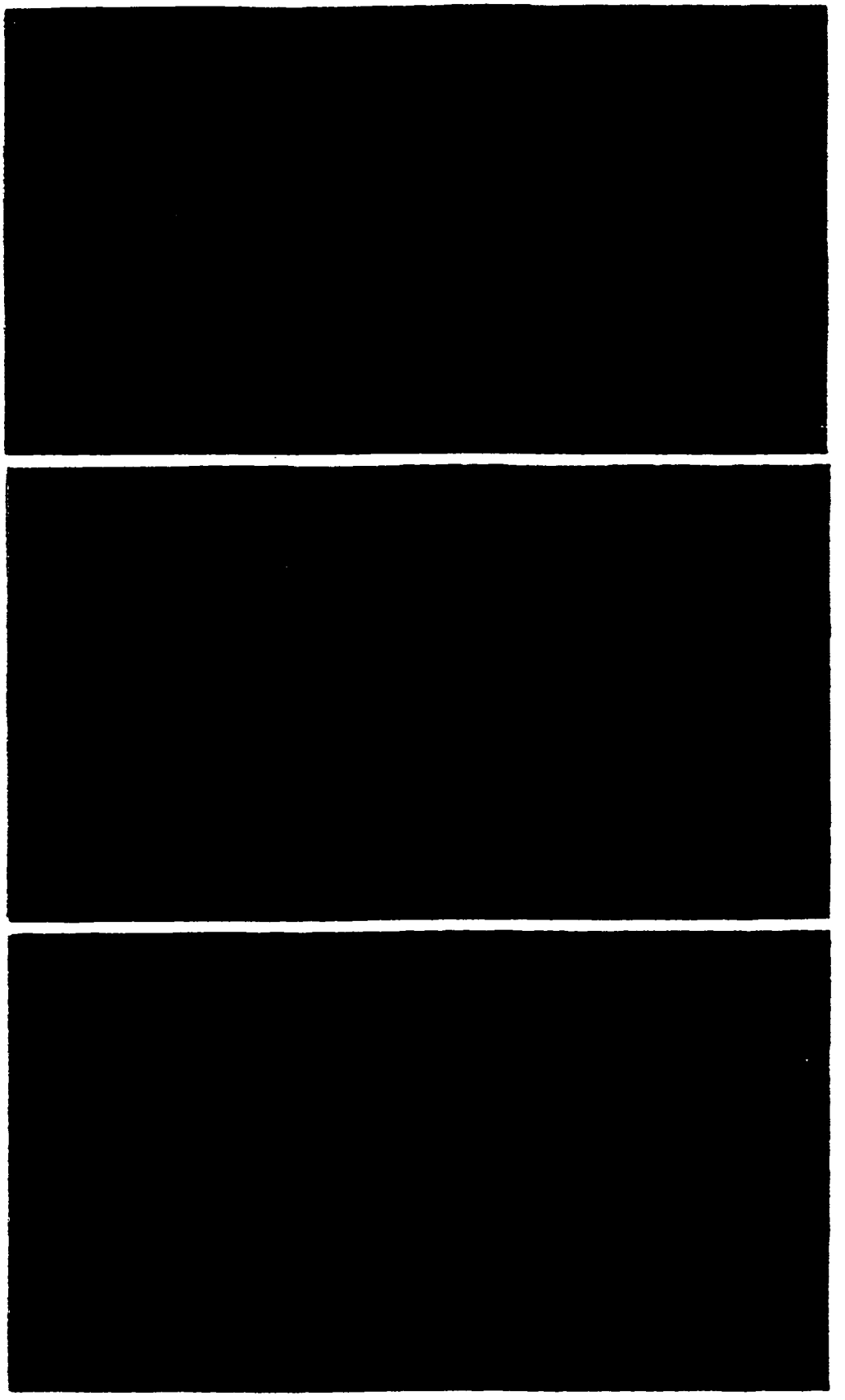

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Plate 5.14. Histological sections of lung tissue from A/J mice inoculated intratracheally with a sublethal dose (10^6 CFU) of *L. pneumophila* 48 h post-challenge. A/J mice were challenged with either live or heat-killed *L. pneumophila*. Hematoxylin and eosin stain.
A) Control lung from A/J mouse inoculated intratracheally with DPBS (X 255). B) A/J mouse lung challenged with heat-killed *L. pneumophila* (X 255). C) A/J mouse lung infected with live *L. pneumophila*. Note thickened interalveolar septa and increased presence of RBCs (X 255).

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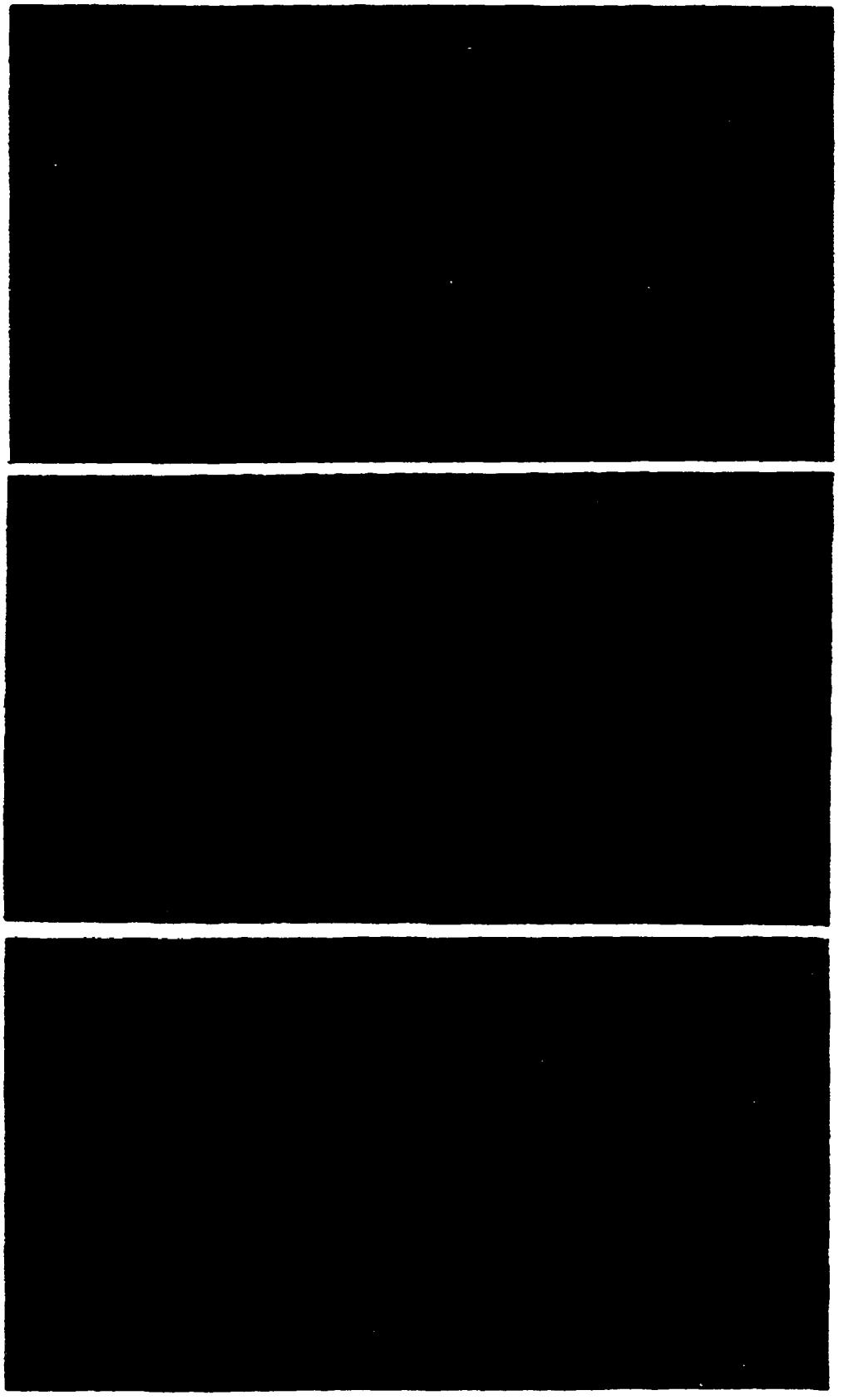

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Plate 5.15. Histological sections of lung tissue from A/J mice inoculated intratracheally with a sublethal dose (10^6 CFU) of *L. pneumophila* 72 h post-challenge. A/J mice were challenged with either live or heat-killed *L. pneumophila*. Hematoxylin and eosin stain.
A) Control lung from A/J mouse inoculated intratracheally with DPBS (X 255). B) A/J mouse lung challenged with heat-killed *L. pneumophila* (X 255). C) A/J mouse lung infected with live *L. pneumophila*. Note marked increase of inflammatory cells, thickened interalveolar septa, limited number of opened alveoli, fibrin deposits and increased presence of RBCs. Both cellular and protein-rich exudate are present (X 255).

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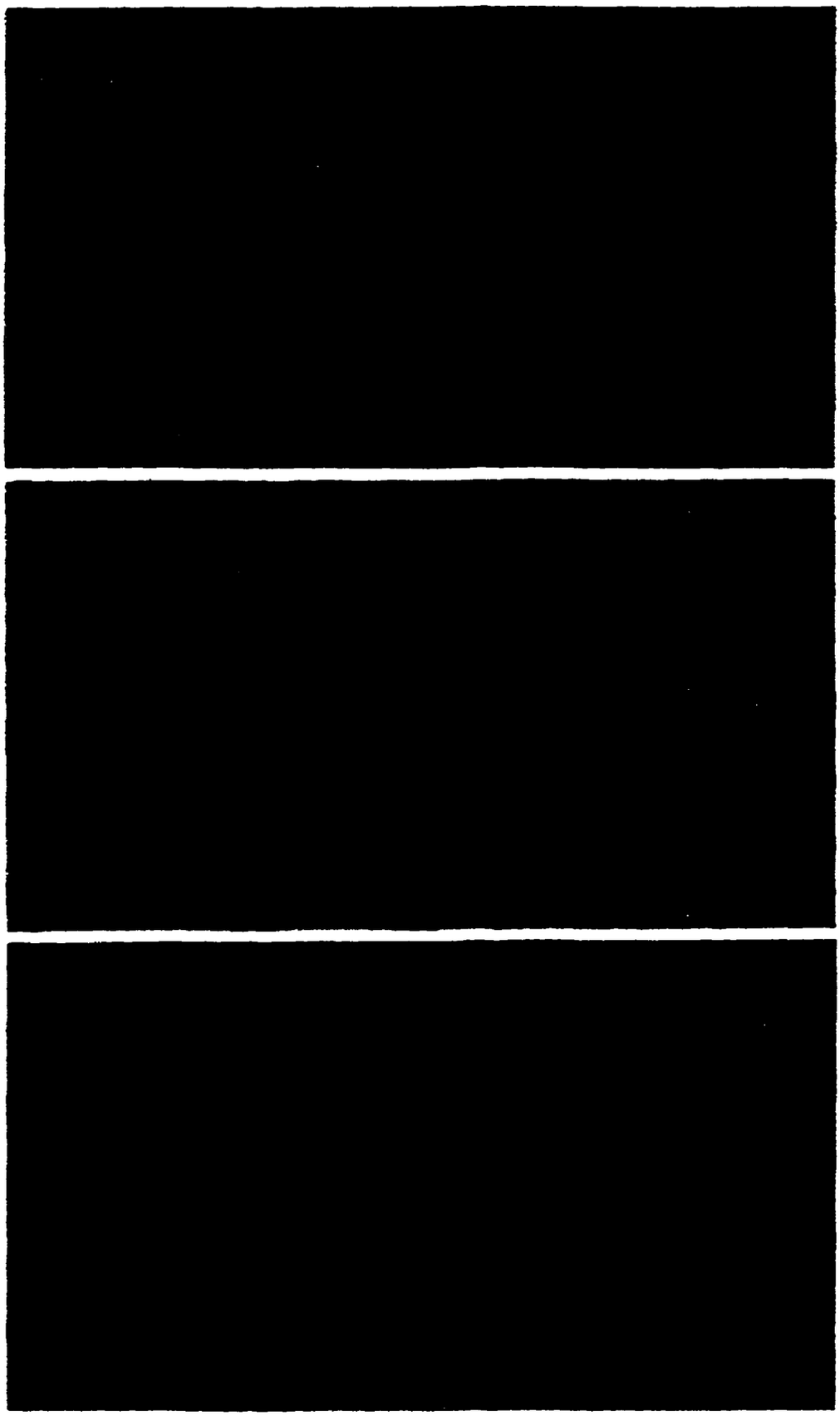

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Plate 5.16. Histological sections of lung tissue from A/J mice inoculated intratracheally with a sublethal dose (10^6 CFU) of *L. pneumophila* 96 h post-challenge. A/J mice were challenged with either live or heat-killed *L. pneumophila*. Hematoxylin and eosin stain.
A) Control lung from A/J mouse inoculated intratracheally with DPBS (X 255). B) A/J mouse lung challenged with heat-killed *L. pneumophila* (X 255). C) A/J mouse lung infected with live *L. pneumophila*. Note marked increase of inflammatory cells, thickened interalveolar septa, limited number of opened alveoli, fibrin deposits and increased presence of RBCs. Both cellular and protein-rich exudate are present. Terminal bronchioles are filled with cellular exudate (X 255).

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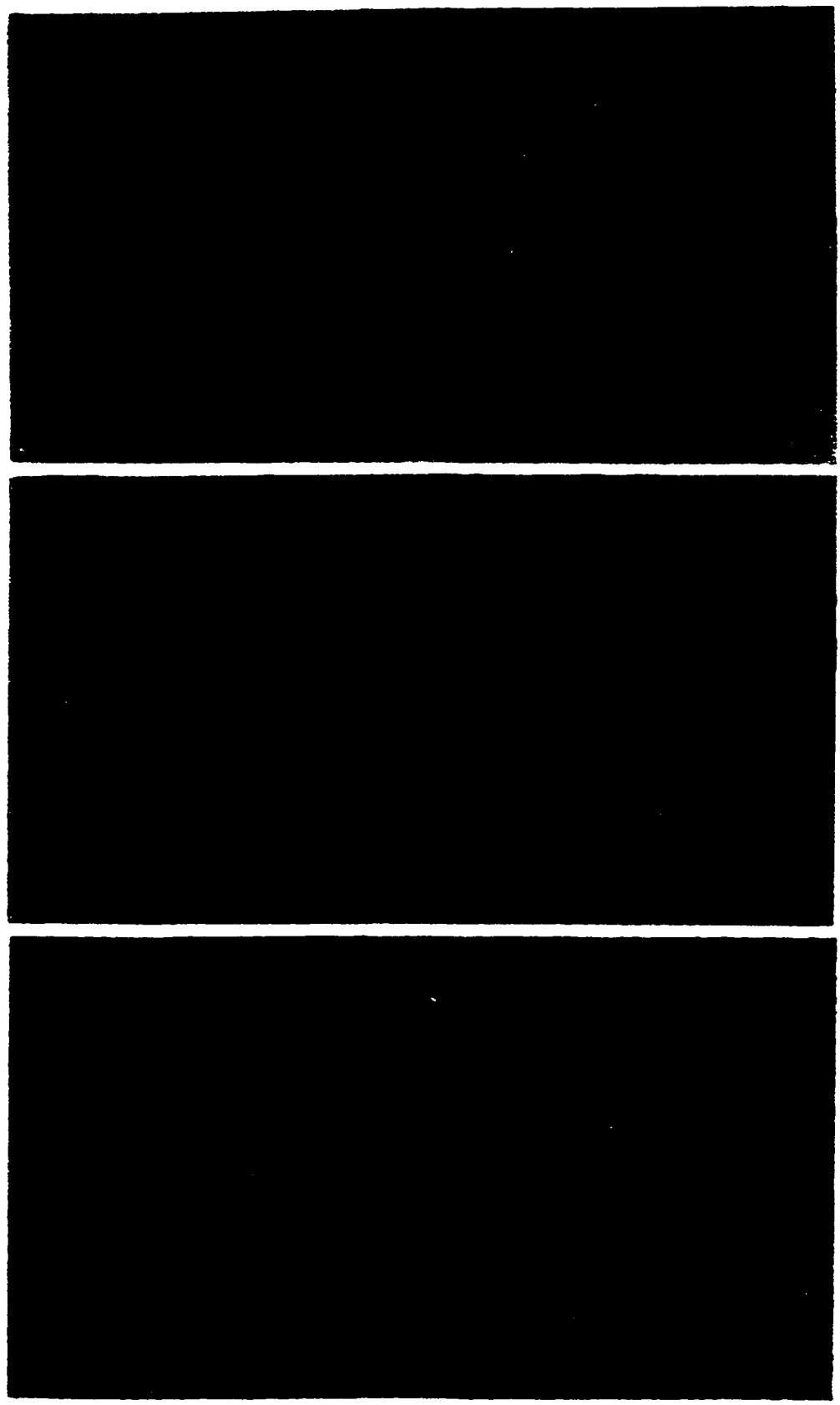

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Plate 5.17. Histological sections of lung tissue from A/J mice inoculated intratracheally with a sublethal dose (10^6 CFU) of *L. pneumophila* 120 h post-challenge. A/J mice were challenged with either live or heat-killed *L. pneumophila*. Hematoxylin and eosin stain.
A) Control lung from A/J mouse inoculated intratracheally with DPBS (X 255). B) A/J mouse lung challenged with heat-killed *L. pneumophila* (X 255). C) A/J mouse lung infected with live *L. pneumophila*. Note increase of inflammatory cells, thickened interalveolar septa, limited number of opened alveoli, and increased presence of RBCs. Both cellular and protein-rich exudate are present (X 255).

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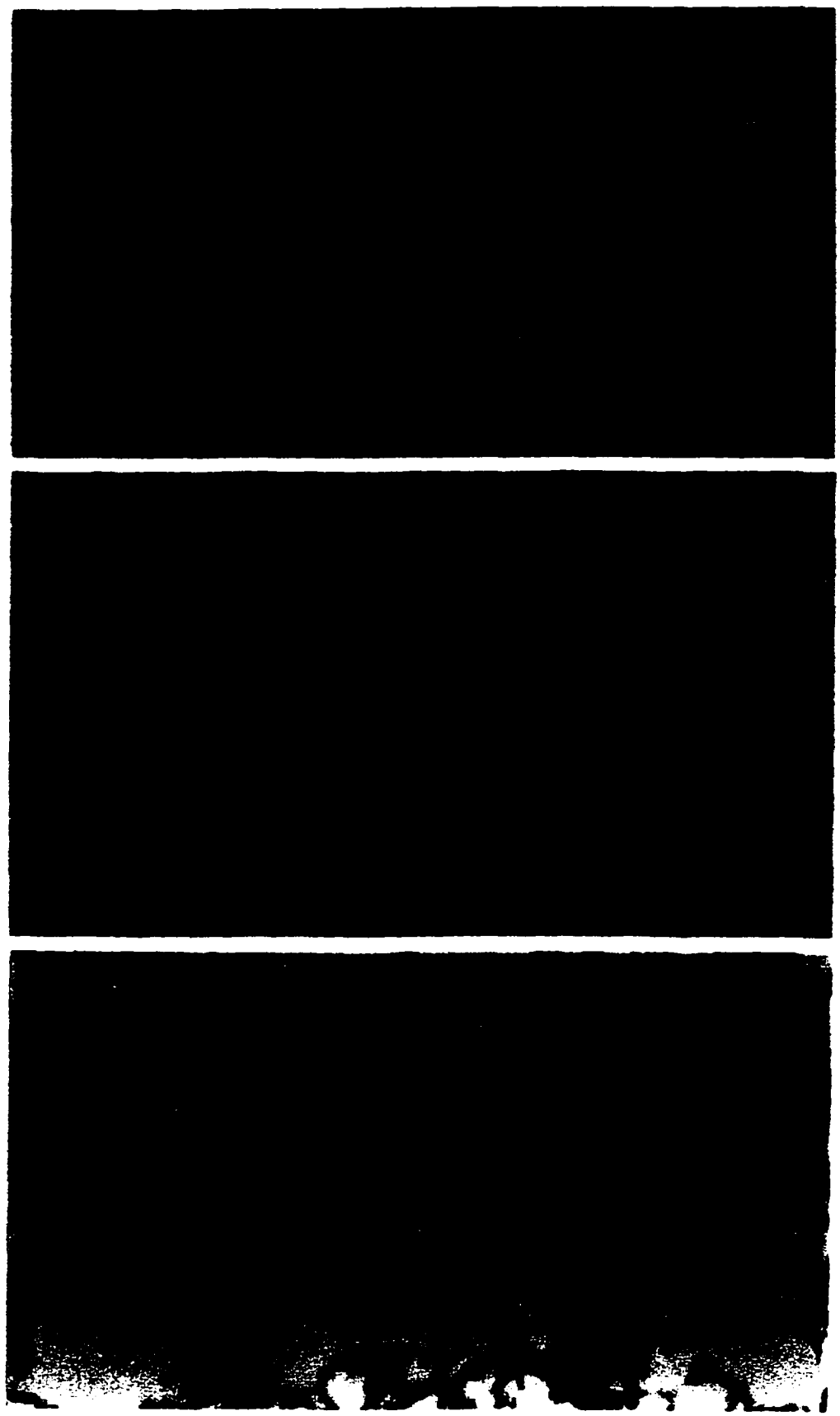

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pneumophila. Plates 5.7 through 5.9 depict the gross pathology associated with lungs of A/J mice treated with heat killed *L. pneumophila* while plates 5.10 to 5.17 show the histopathological appearance of lungs. There was relatively no pathological or histopathological changes associated with challenge of A/J mice with heat-killed *L. pneumophila.*

5.4.3 **Cytokine determination from murine specimens following sublethal challenge.** The profiles of IL-1β ,TNF-α, MIP-2, IL-10, IL-12 (p40 and p70) and IFN-γ from BAL fluids and lung homogenates of A/J mice were examined immediately following sublethal intratracheal challenge with *L. pneumophila* N7 as well as at 12, 24, 48, 72 and 96 h post-infection. The proinflammatory cytokines IL-1β and TNF-α were detected in both BAL fluids and lung homogenates at 12 h post-challenge with live *L. pneumophila.* Levels of IL-1β were maximal in BAL fluids at 48 h post-infection (402 ± 86 pg/ml) (Figure 5.2) while maximum levels in lung homogenates were detected at 72 h post-infection (4029 ± 1688 pg/ml) (Figure 5.3). Maximal levels of TNF-α were detected at 48 h in both BAL fluids (Figure 5.5) and lung homogenates (Figure 5.6) (1973 ± 238 pg/ml and 4422 ± 1677 pg/ml respectively). The C-X-C chemokine, MIP-2, a potent activator of neutrophil activation and a potent neutrophil attractant, reached maximum levels in BAL fluids at 24 h post-challenge (331 ± 69 pg/ml) (Figure 5.8) and in lung

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homogenates at 48 h (2800 ± 267 pg/ml) (Figure 5.9). As IL-12 is an important mediator of cell-mediated host responses, the ability of A/J mice to produce localized IL-12 during *L. pneumophila* infection was evaluated. IL-12 p40 reached maximum levels in BAL fluids 72 h post-infection (824 ± 64 pg/ml) (Figure 5.11) and in lung homogenates by 48 h post-challenge (3868 ± 2208 pg/ml) (Figure 5.12). In addition, the heterodimeric, biologically active form of IL-12, p70 protein, reached maximum levels in BAL fluids (Figure 5.14) and in lung homogenates (Figure 5.15) at 48 h post-challenge (415 ± 136 pg/ml and 958 ± 583 pg/ml respectively). IFN-γ reached peak levels at 72 h post-infection in both BAL fluids (2345 ± 555 pg/ml) (Figure 5.17) and lung homogenates (1579 ± 432 pg/ml) (Figure 5.18). IL-10 was not detected in either BAL fluids or lung homogenates following *L. pneumophila* challenged (Figure 5.20 and 5.21 respectively)

In contrast, the levels of cytokines detected in serum differed from those detected in BAL fluids or lung homogenates. Although IL-1β was induced by *L. pneumophila* infection, it was detected only at low levels and reached a peak at 48 h post-infection (64 ± 20 pg/ml) (Figure 5.4). Furthermore, very low levels of TNF-α were detected in sera of infected A/J mice (Figure 5.7). A significant rise in the levels of IL-12 p40 and IL-12 p70 (Figures 5.13 and 5.16 respectively) were detected in sera from *Legionella*-infected mice as compared to uninfected animals. IL-12 p40 was detected

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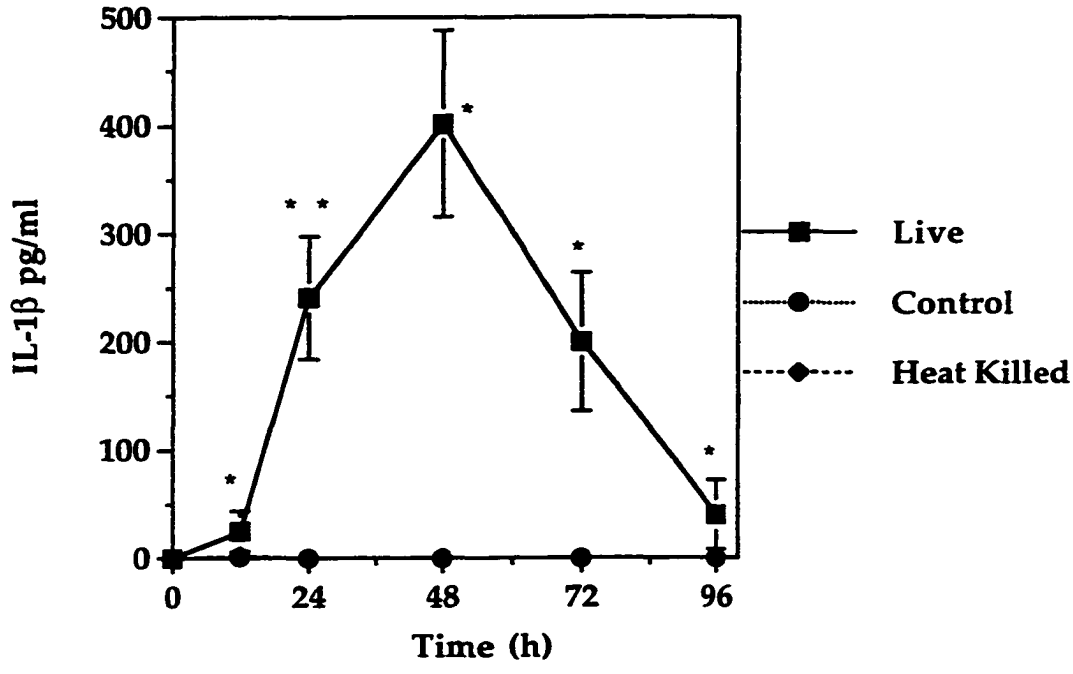

Figure 5.2: Interleukin-1β production from bronchoalveolar lavage fluids of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points and lungs were lavaged with 1 ml sterile DPBS and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of five mice per group at each time point. $*p < 0.0001$ (live vs. heat-killed and control).

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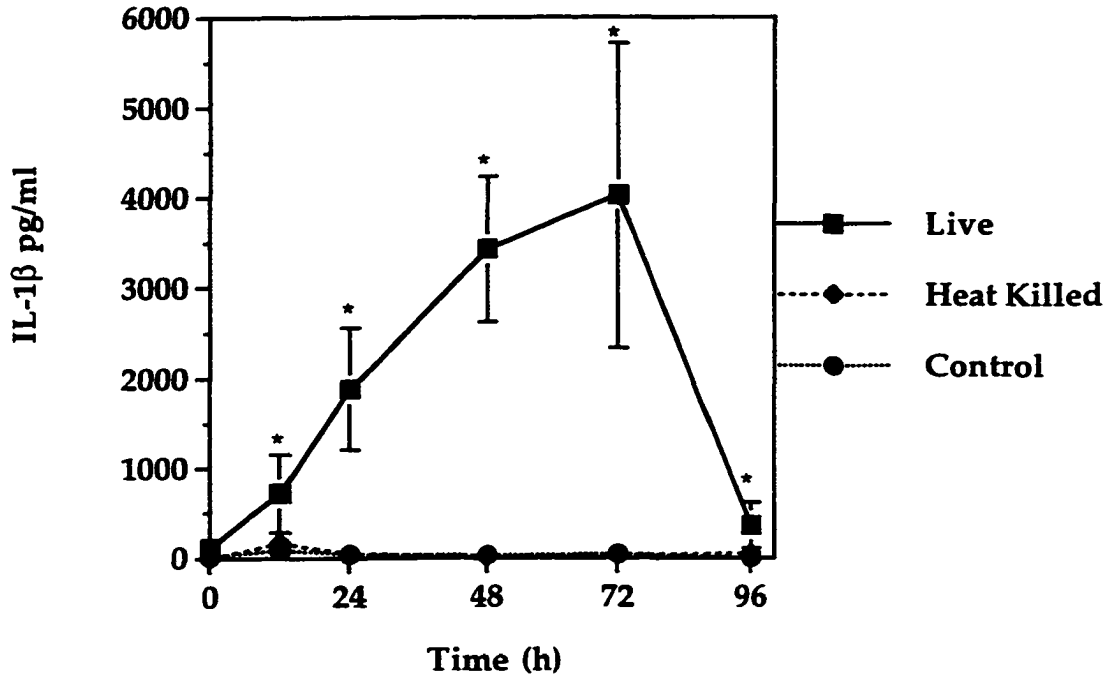

Figure 5.3: Interleukin-1β production from lung homogenates of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points and lungs were removed and homogenized and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of five mice per group at each time point. $^*p < 0.0001$ (live vs. heat-killed and control).

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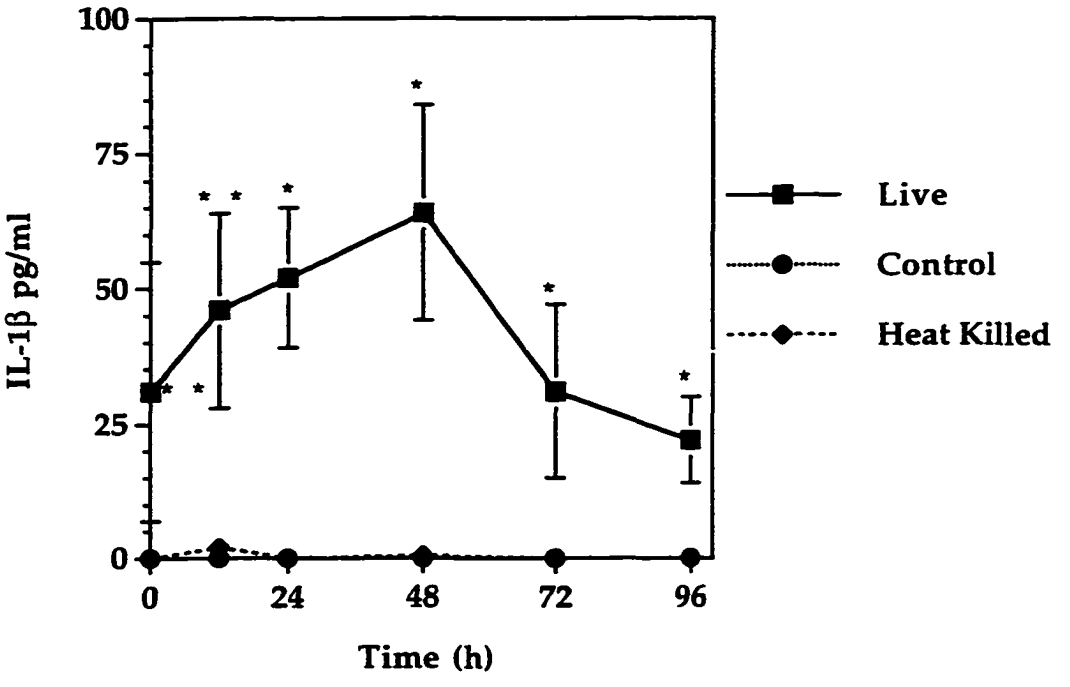

Figure 5.4: Interleukin-1β production from sera of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points, serum was collected and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of four mice per group at each time point. $*p < 0.03$, $**p = 0.0022$ (live vs. heat-killed and control).

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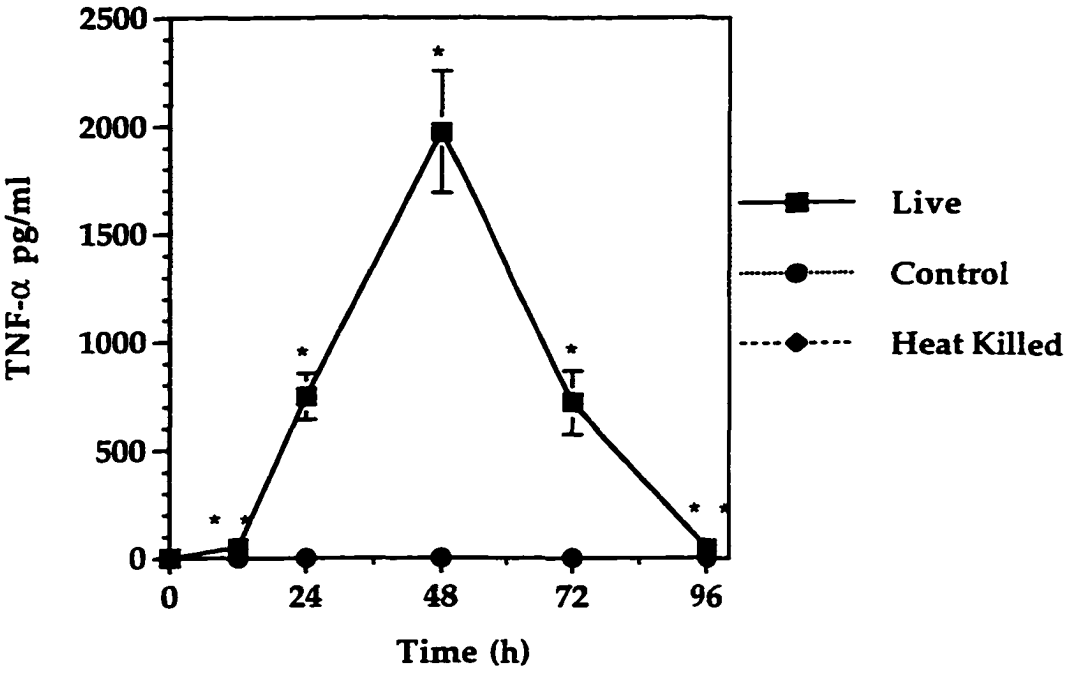

Figure 5.5: Tumor necrosis factor-α production from bronchoalveolar lavage fluids of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points and lungs were lavaged with 1 ml sterile DPBS and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of five mice per group at each time point. $*p < 0.0006$, $**p < 0.03$ (live vs. heat-killed and control).

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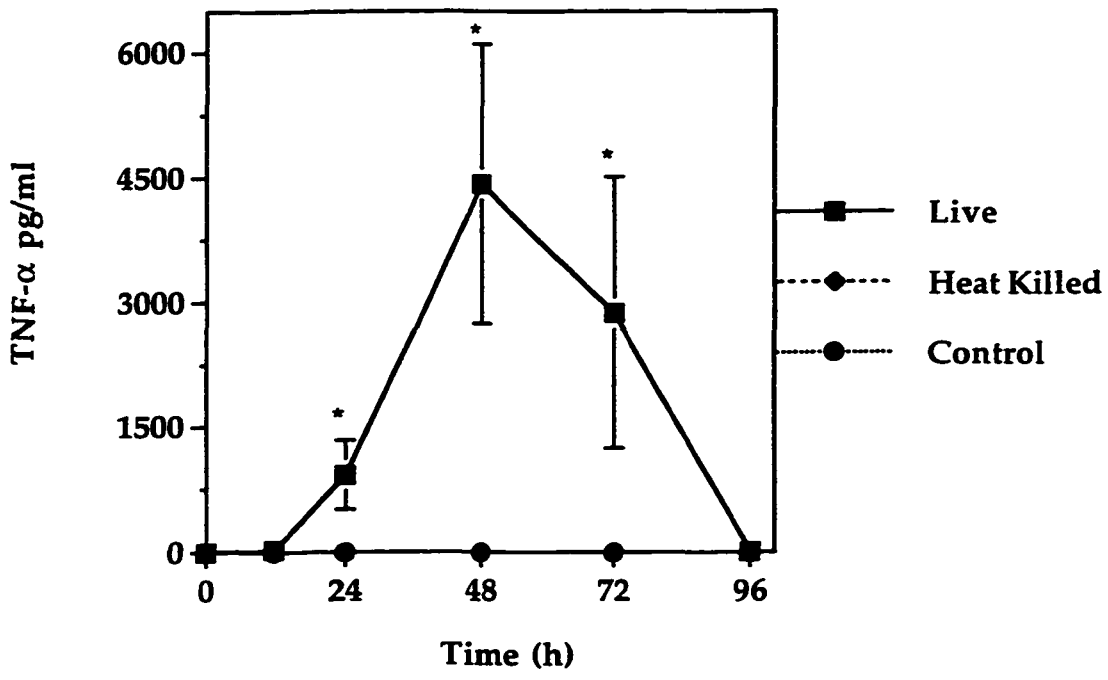

Figure 5.6: Tumor necrosis factor-α production from lung homogenates of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points and lungs were removed and homogenized and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of five mice per group at each time point. $*p < 0.0001$ (live vs. heat-killed and control).

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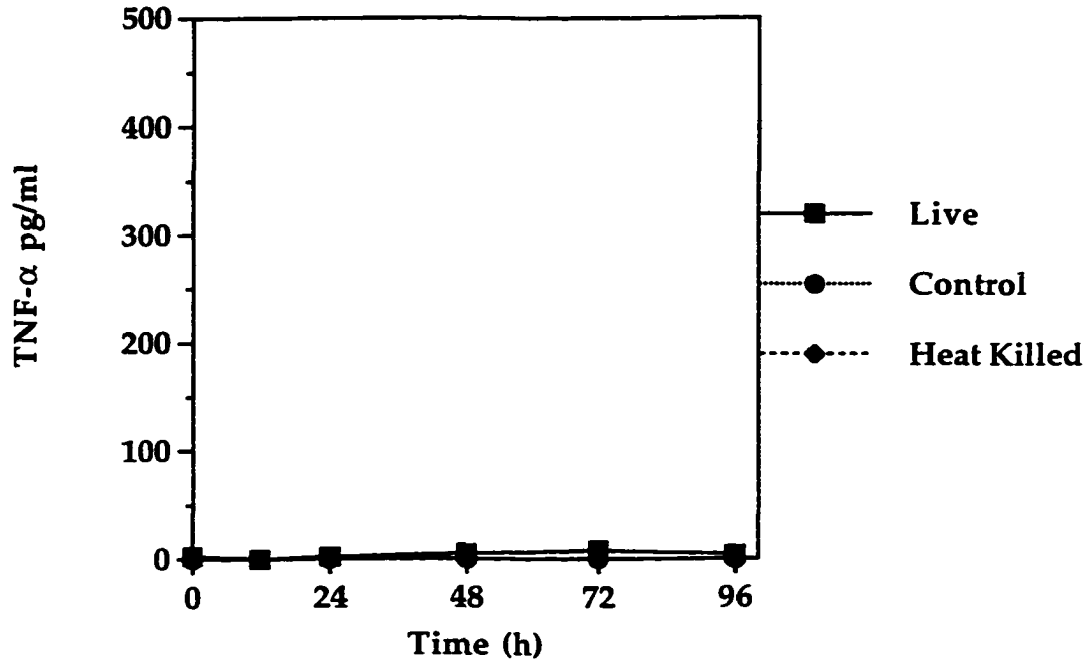

Figure 5.7: Tumor necrosis factor-α production from sera of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points, serum was collected and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of four mice per group at each time point.

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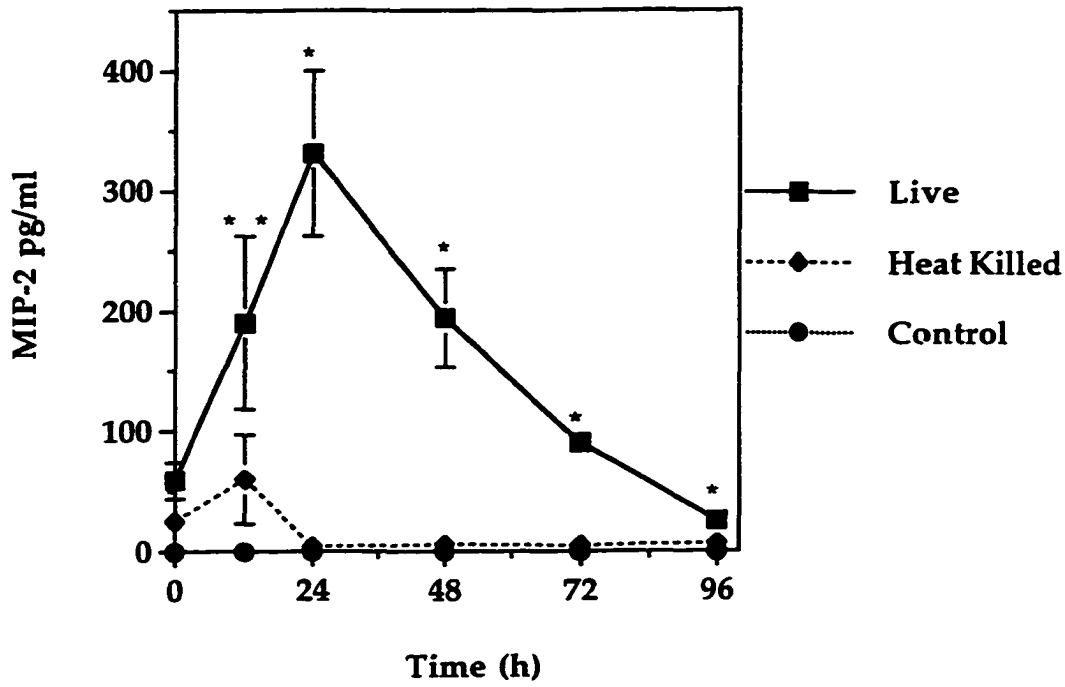

Figure 5.8: Macrophage inflammatory protein-2 production from bronchoalveolar lavage fluids of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points and lungs were lavaged with 1 ml sterile DPBS and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of four mice per group at each time point. $^*p = 0.0002$, $^{**}p = 0.0207$ (live vs. heat-killed and control).

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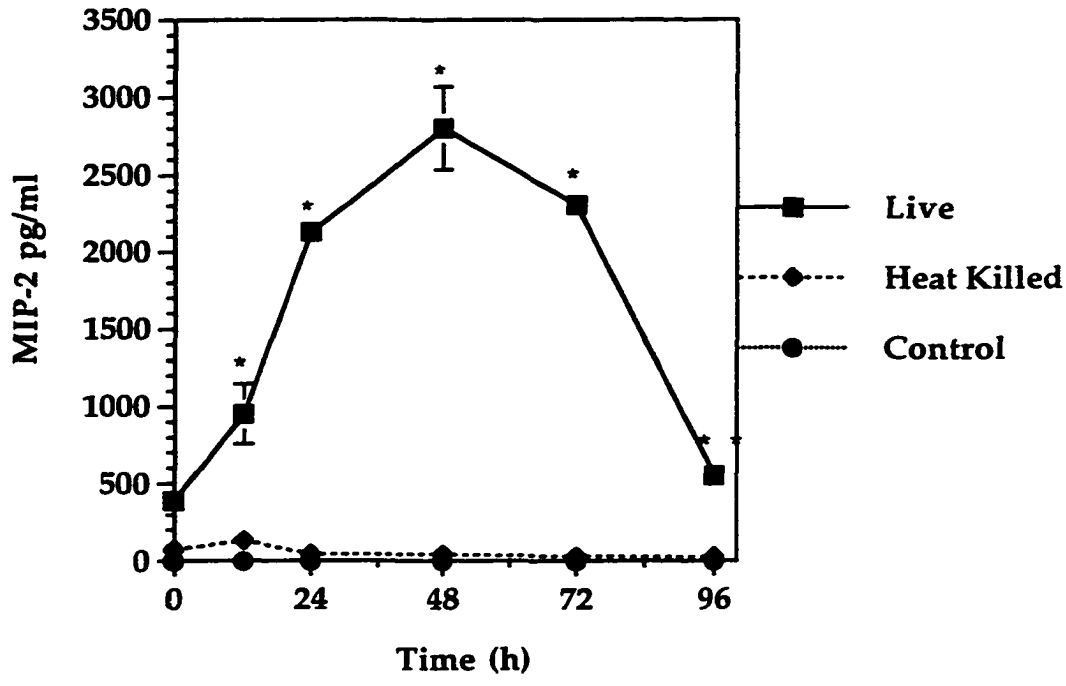

Figure 5.9: Macrophage inflammatory protein-2 production from lung homogenates of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points and lungs were removed and homogenized and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of four mice per group at each time point. $*p < 0.0001$, $**p = 0.002$ (live vs. heat-killed and control).

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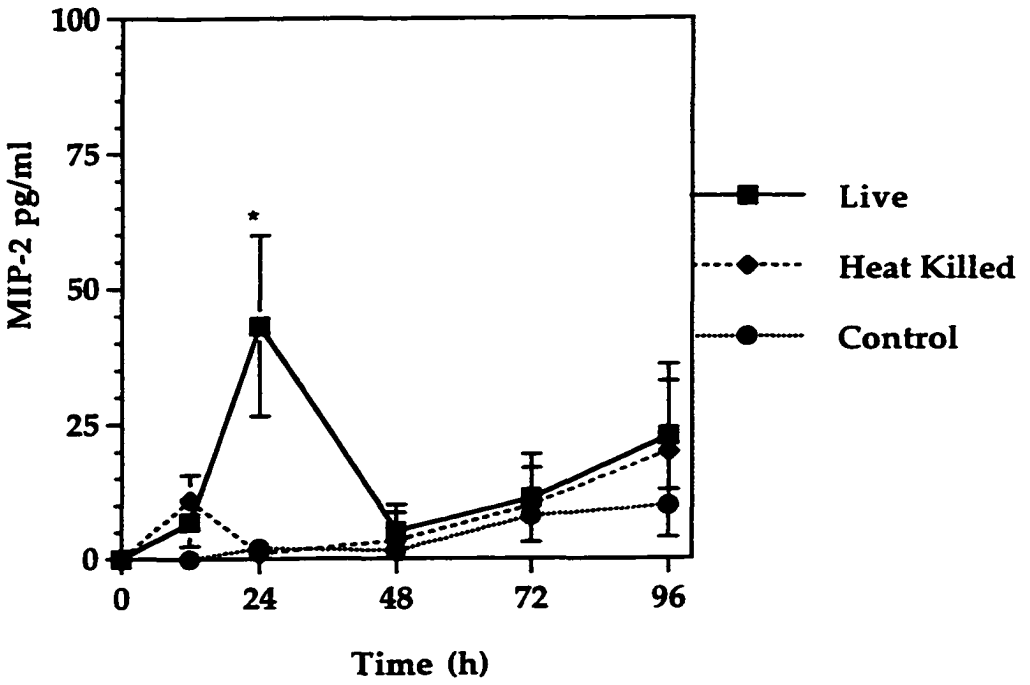

Figure 5.10: Macrophage inflammatory protein-2 production from sera of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points, serum was collected and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of three mice per group at each time point. $*p < 0.0001$ (live vs. heat-killed and control).

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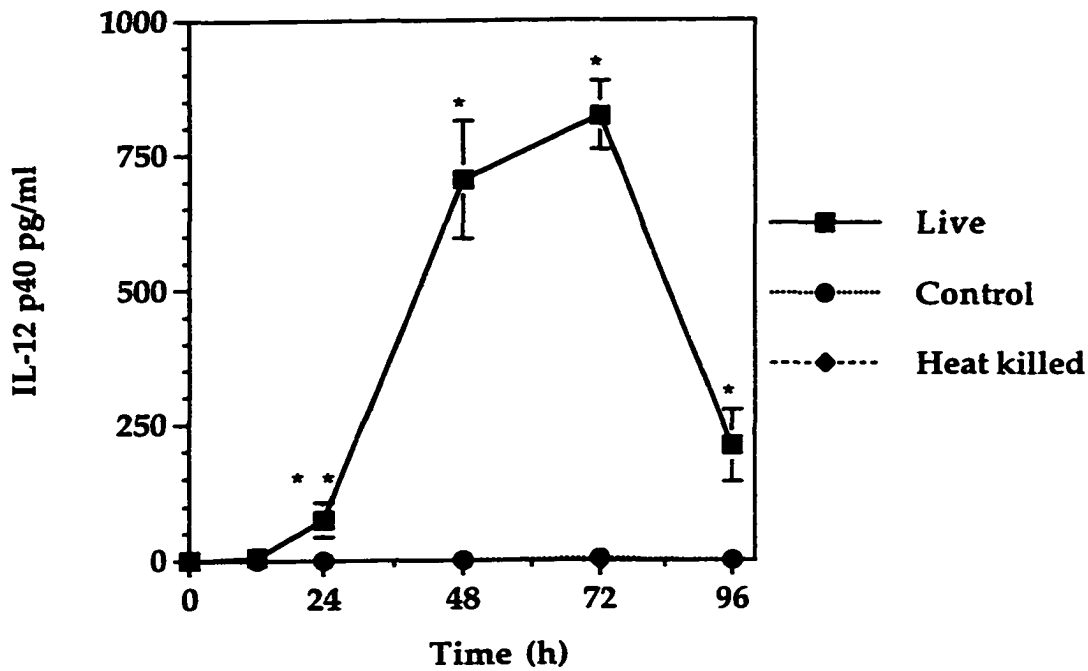

Figure 5.11: Interleukin-12 p40 production from bronchoalveolar lavage fluids of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points and lungs were lavaged with 1 ml sterile DPBS and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of five mice per group at each time point. $*p < 0.0001$, $**p = 0.008$ (live vs. heat-killed and control).

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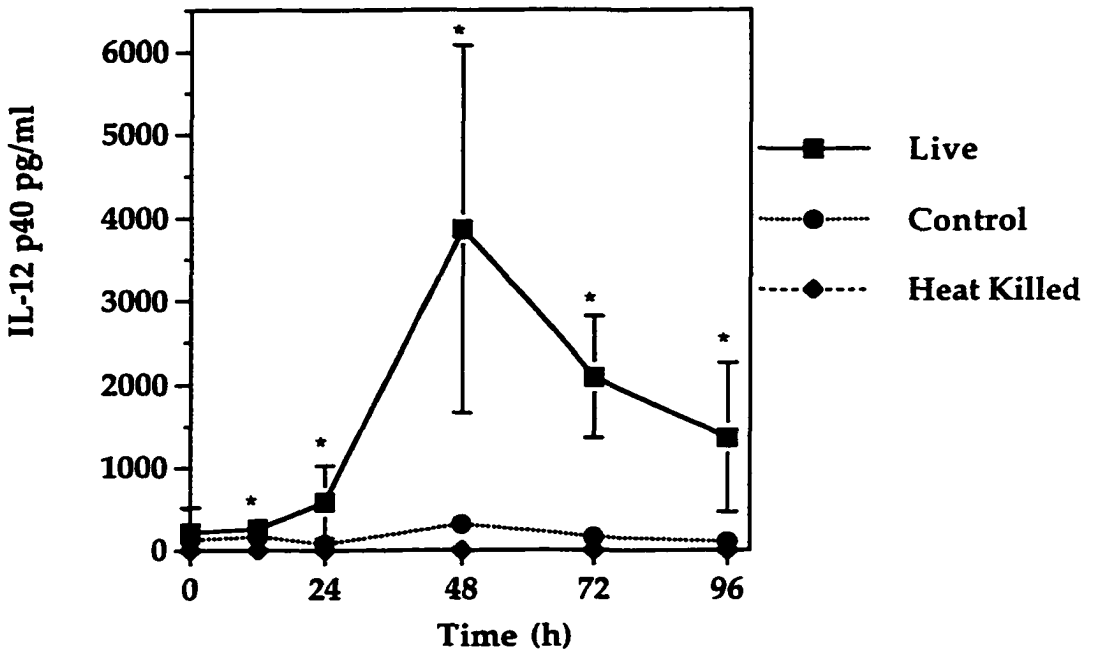

Figure 5.12: Interleukin-12 p40 production from lung homogenates of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points and lungs were removed and homogenized and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of five mice per group at each time point. $^*p < 0.0001$ (live vs. heat-killed and control).

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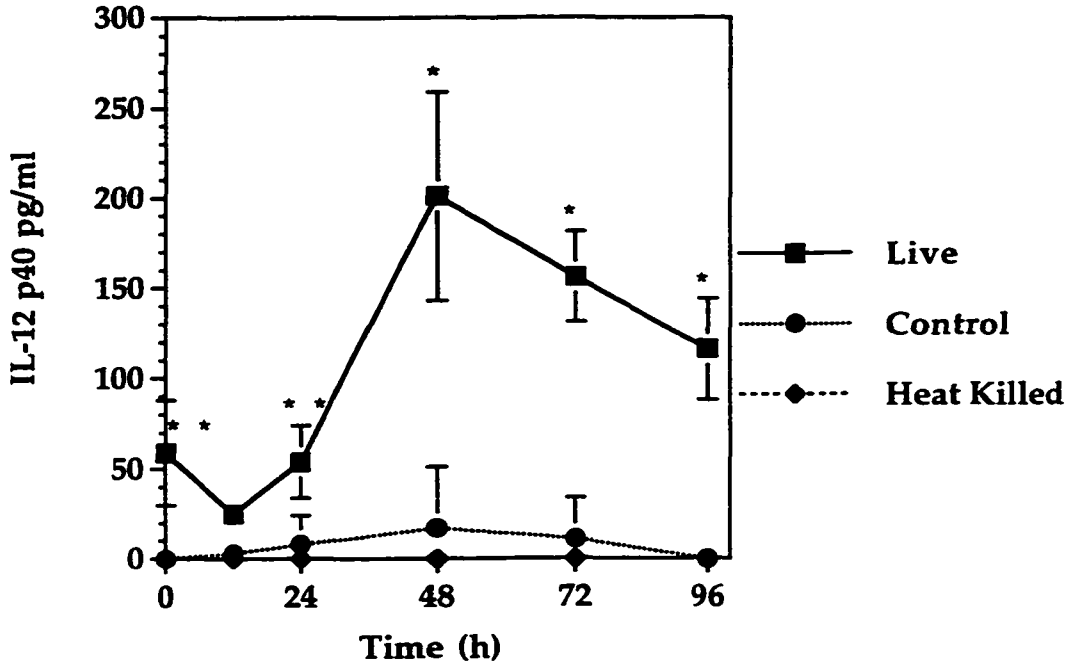

Figure 5.13: Interleukin-12 p40 production from sera of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points, serum was collected and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of four mice per group at each time point. $*p < 0.0002$, $**p < 0.03$ (live vs. heat-killed and control).

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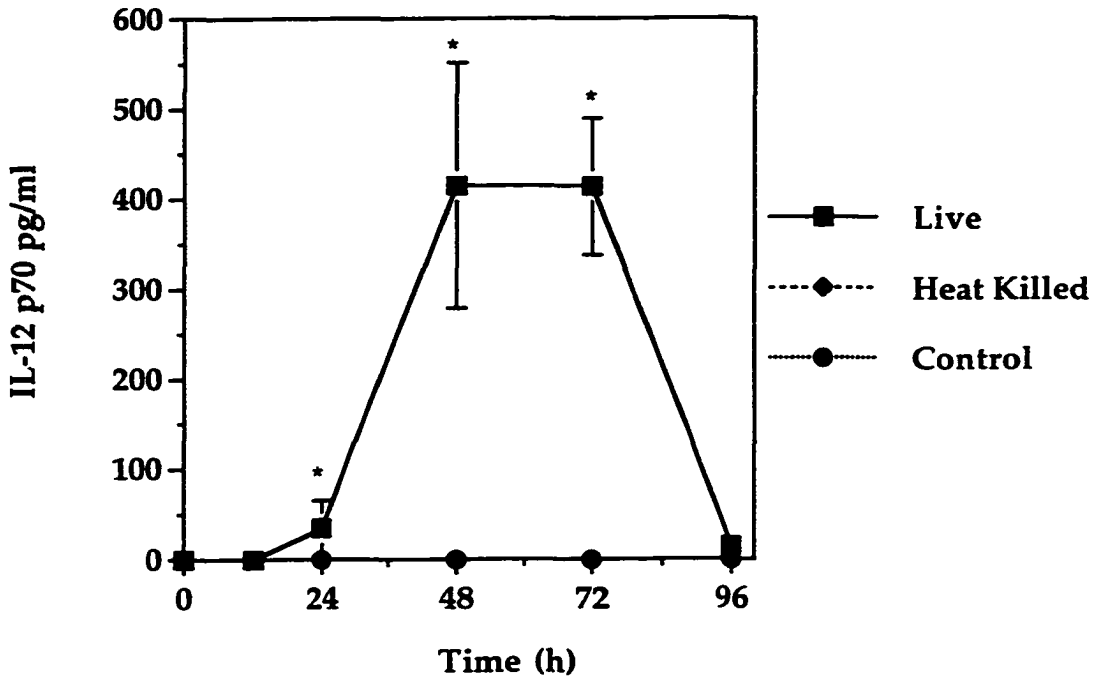

Figure 5.14: Interleukin-12 p70 production from bronchoalveolar lavage fluids of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points and lungs were lavaged with 1 ml sterile DPBS and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of five mice per group at each time point. $^*p$ = 0.0002 (live vs. heat-killed and control).

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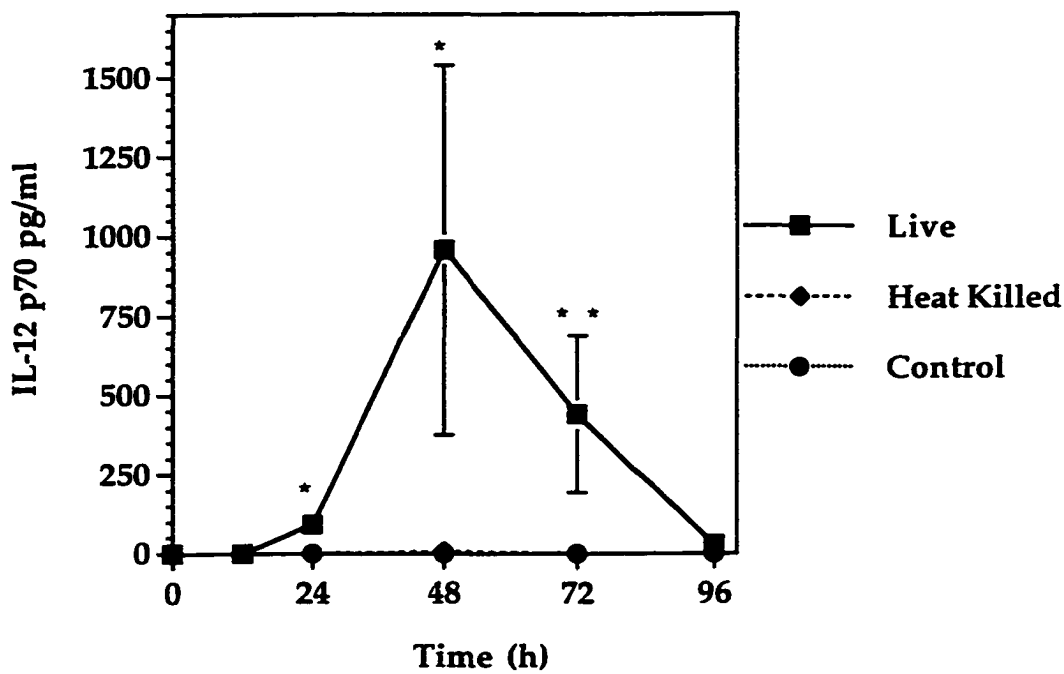

Figure 5.15: Interleukin-12 p70 production from lung homogenates of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points and lungs were removed and homogenized and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of five mice per group at each time point. $*p = 0.0022$, $**p = 0.0095$ (live vs. heat-killed and control).

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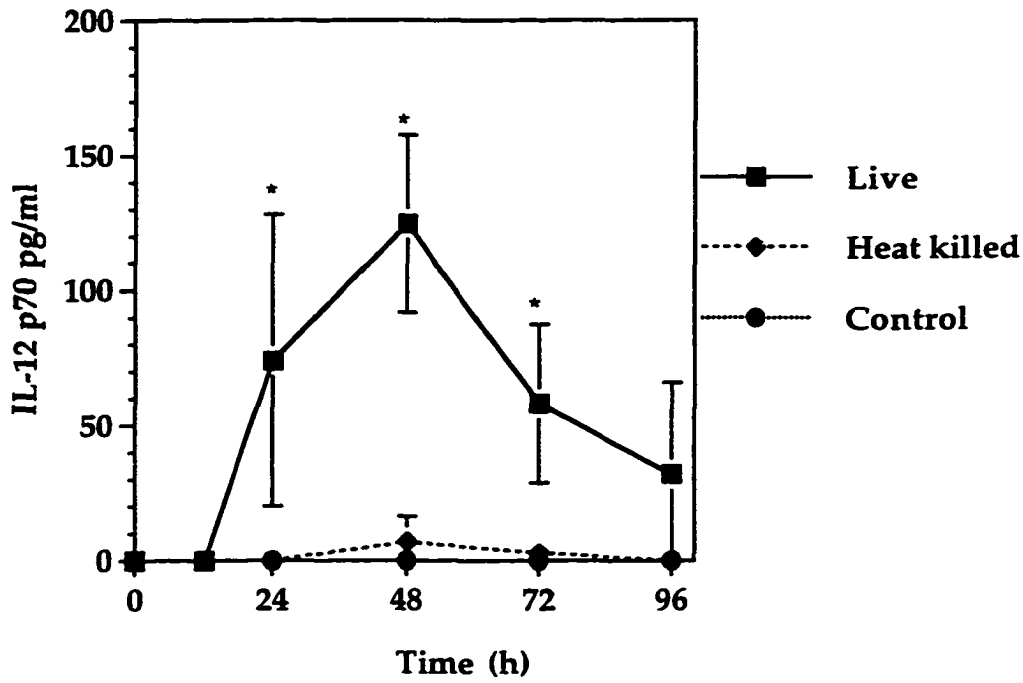

Figure 5.16: Interleukin-12 p70 production from sera of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points, serum was collected and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of four mice per group at each time point. $*p < 0.0001$ (live vs. heat-killed and control).

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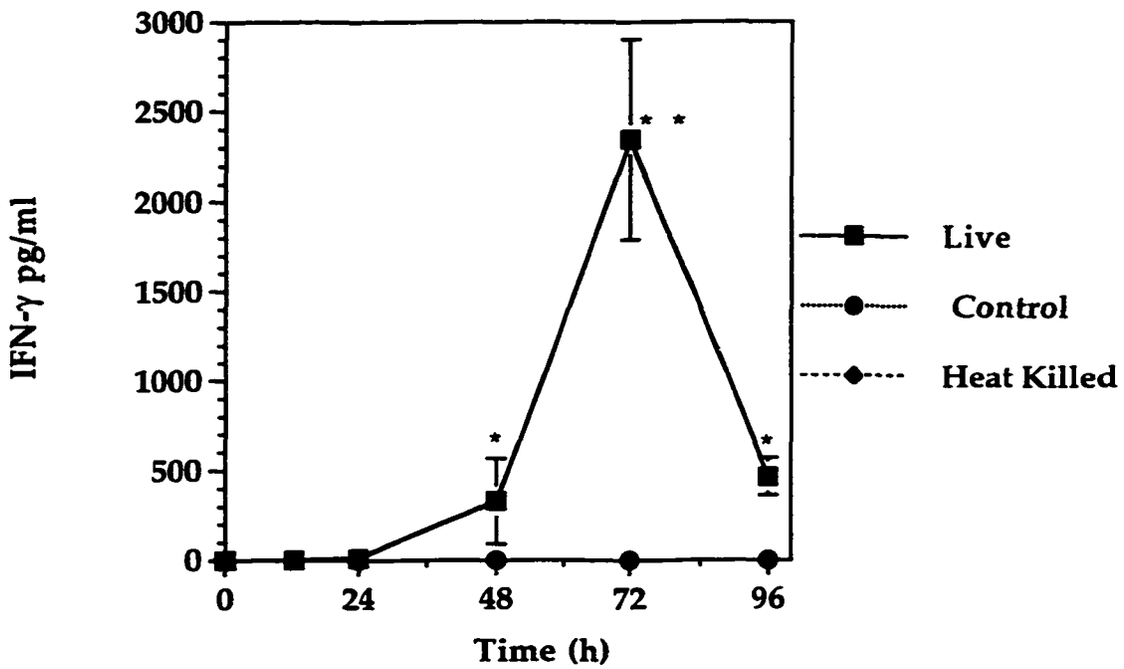

Figure 5.17: Interferon-γ production from bronchoalveolar lavage fluids of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points and lungs were lavaged with 1 ml sterile DPBS and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of five mice per group at each time point. $*p < 0.008$, $**p < 0.0001$ (live vs. heat-killed and control).

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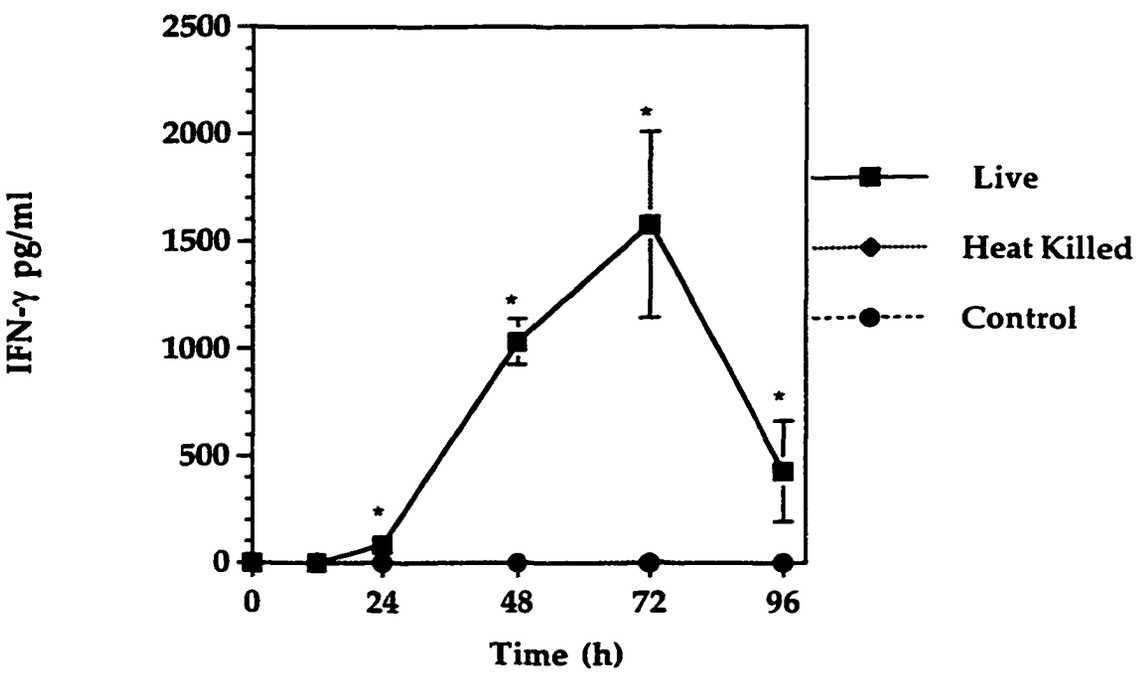

Figure 5.18: Interferon-γ production from lung homogenates of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points and lungs were removed and homogenized and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of five mice per group at each time point. $*p < 0.0001$ (live vs. heat-killed and control).

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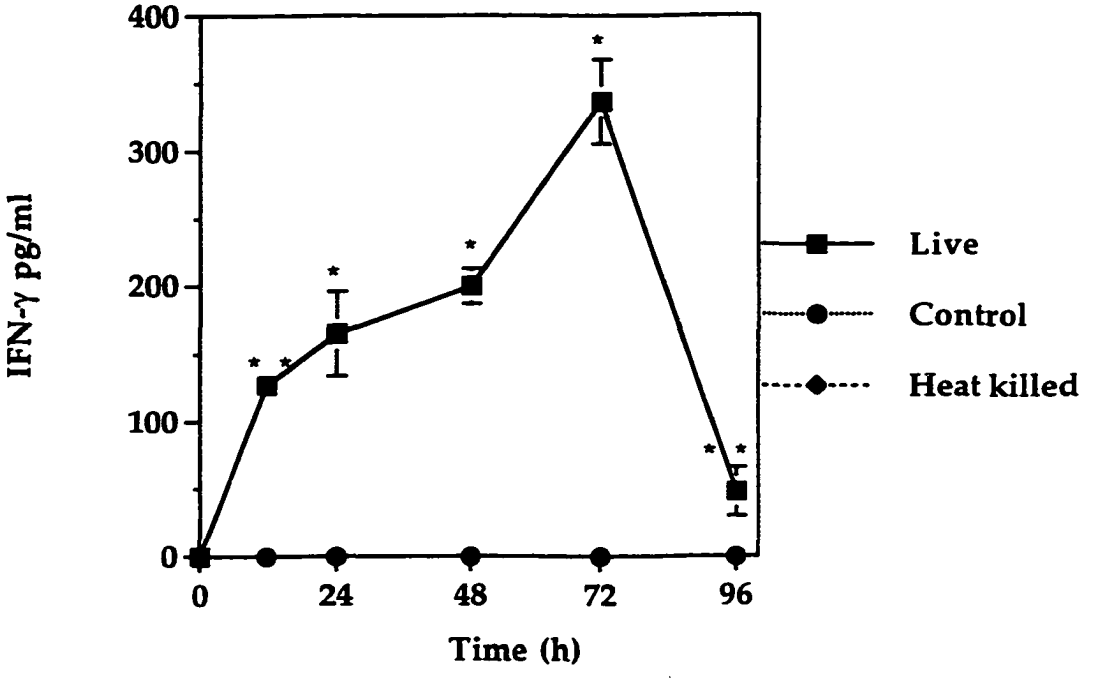

Figure 5.19: Interferon-γ production from sera of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points, serum was collected and assayed for cytokine production by ELISA. Results are expressed as means ± standard deviations from three separate experiments each consisting of four mice per group at each time point. $*p < 0.0001$, $**p = 0.0022$ (live vs. heat-killed and control).

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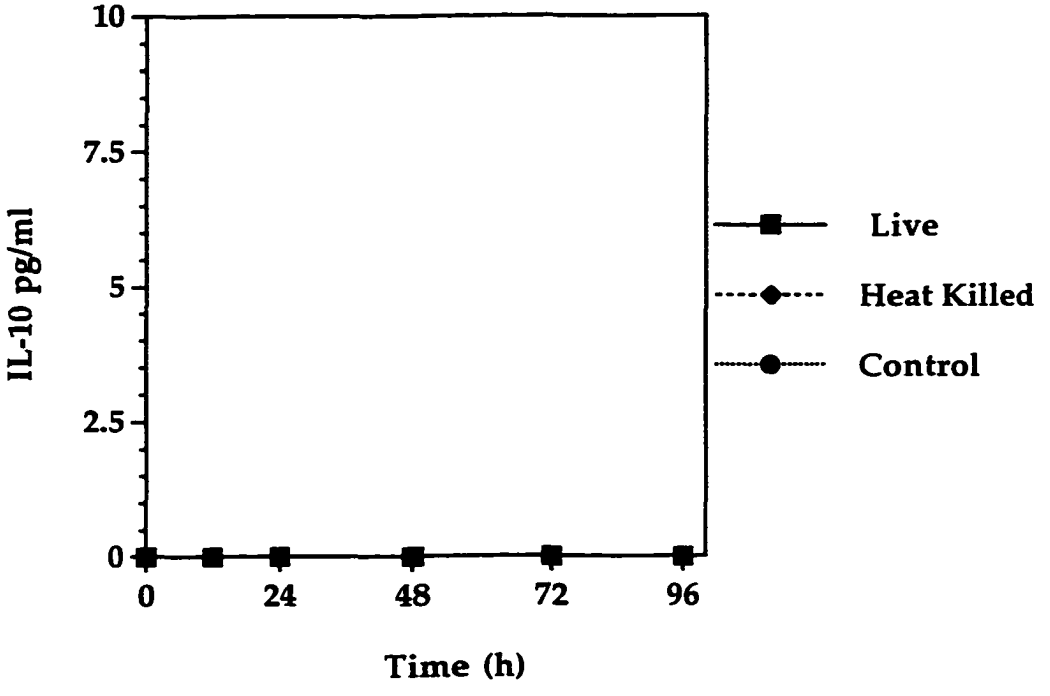

Figure 5.20: Interleukin-10 production from bronchoalveolar lavage fluids of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points and lungs were lavaged with 1 ml sterile DPBS and assayed for cytokine production by ELISA. Results are expressed as means from three separate experiments each consisting of three mice per group at each time point.

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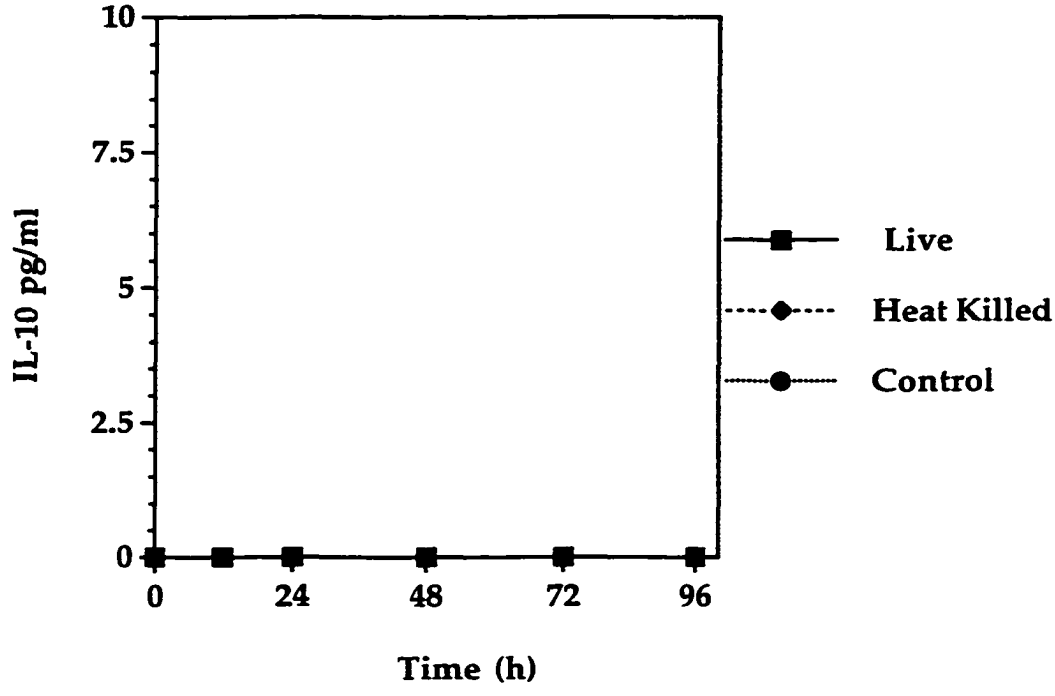

Figure 5.21: Interleukin-10 production from lung homogenates of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points and lungs were removed and homogenized and assayed for cytokine production by ELISA. Results are expressed as means from three separate experiments each consisting of four mice per group at each time point.

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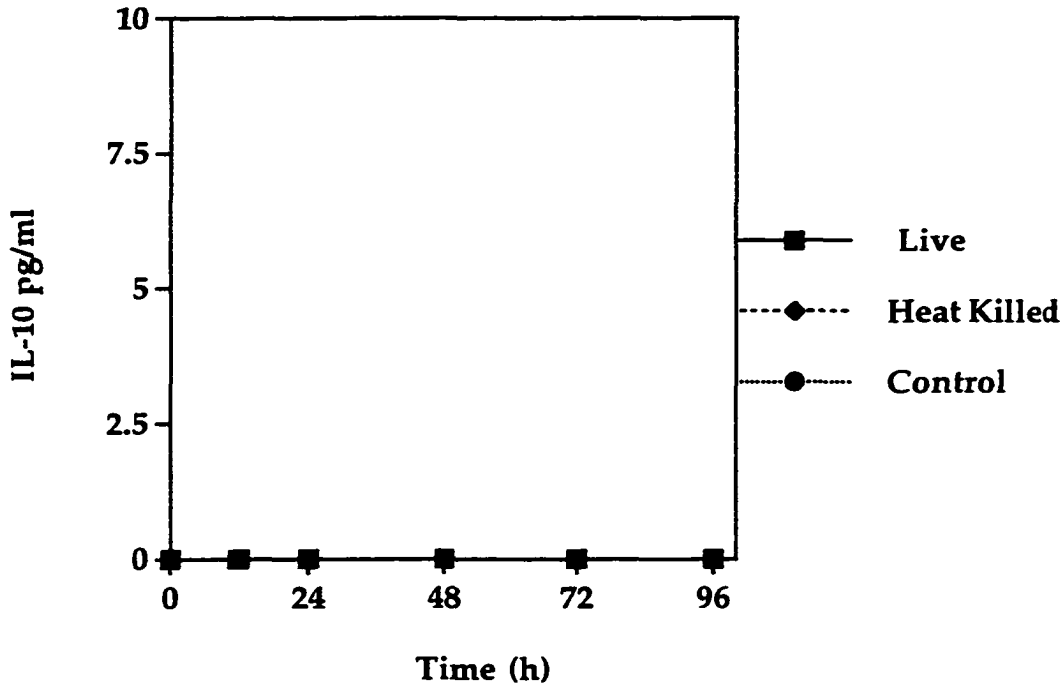

Figure 5.22: Interleukin-10 production from sera of A/J mice challenged with either live or heat-killed *L. pneumophila*. A/J mice were inoculated intratracheally with *L. pneumophila* ($1x10^6$ bacteria per mouse), mice were sacrificed at the indicated time points, serum was collected and assayed for cytokine production by ELISA. Results are expressed as means from three separate experiments each consisting of four mice per group at each time point.

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immediately after surgery. Levels declined during the next 12 h then rose steadily to reach maximum levels 48 h post-infection (201 ± 58 pg/ml). For IL-12 p70, peak levels were also detected at 48 h post-infection (125 ± 33 pg/ml). IFN-γ levels in the sera of mice were initially detected at 12 h post-infection and these rose steadily to reach peak levels at 72 h post-infection (337 ± 31 pg/ml) (Figure 5.19). IL-10 was not detected in the sera from either infected or uninfected mice (Figure 5.22). A/J mice challenged with heat-killed *L. pneumophila* did not produce detectable levels of any of the tested cytokines from BAL fluids, lung homogenates or serum.

5.5 Discussion

These *in vivo* studies have confirmed that *L. pneumophila* is able to replicate within the lungs of A/J mice. Intratracheal administration of a sublethal dose of *L. pneumophila* resulted in evident gross pathologic as well as histopathologic changes in the lung. Microscopic evaluation of lung tissue from *L. pneumophila*-infected mice presented with thickening of interalveolar septa, an increase in red blood cells present and evidence of both cellular and protein-rich exudate that extended to the terminal bronchioles during later time points. Despite the ability of A/J mice to resolve a sublethal *L. pneumophila* infection, pathology was associated with lung specimens even after organism clearance. During human disease *L. pneumophila* is atypical among pneumonic pathogens in that a slow rate of radiographic

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resolution is shown (110). This frequently lags behind clinical recovery; thus, the observation that there was pathology seen in mouse lungs even after organism clearance may be typical for *L. pneumophila* infections in this mouse model and in humans.

During *L. pneumophila* infection, A/J mice were able to mount a protective response to eradicate four orders of magnitude less than the maximum level of *L. pneumophila* in the lung. This was evidenced by the observation that *L. pneumophila* was cleared from lungs during later stages of infection. Therefore, this model provides insight into an immunocompetent host response, in which a protective immune response "naturally" develops in A/J mice and allows resolution of infection. The mechanism of the response which allows for the development of *L. pneumophila* specific immune responses *in vivo* is unknown. Therefore, the focus of this study was to further investigate the production of the diverse classes of cytokine molecules in the A/J mouse model of Legionnaires' disease. *L. pneumophila* induced production of the proinflammatory cytokines IL-1β and TNF-α and these cytokines were detected in both BAL fluids and lung homogenates during intracellular replication of *L. pneumophila*. Challenge of mice with heat-killed organisms failed to evoke a potent IL-1β or TNF-α response as induced cytokine levels were similar to those of uninfected control animals. It is well known that IL-1β and TNF-α

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are crucial to host immunity. One important role for these molecules is to influence the localization of the inflammatory response to the site of infection by facilitating cell infiltration and up-regulation of cell adhesion molecules, thus setting the stage for immune cell migration into infected tissue (112, 216, 81). In this study the highest concentration of IL-1β and TNF-α were observed in BAL fluids and lung homogenates indicating that the primary source of these molecules was from this site while, the serum levels of IL-1β and TNF-α were low. Similar observations by Yu *et al.* (372) were made for site-specific elevated levels of TNF-α in the lungs of mice following repeated respiratory challenge with *Pseudomonas aeruginosa* while low levels of TNF-α were detected in the sera of mice. It has been observed *in vitro* that TNF-α appears to play an important role in limiting *L. pneumophila* infection (37, 314). The data suggest that the process by which A/J mice resolve infection may be related, in part, to a potent TNF-α response. However, the precise mechanisms by which these animals produce TNF-α in response to live *L. pneumophila* and the role this cytokine plays in infection require further study.

A potent cellular infiltrate was evident in the lung tissue of *Legionella*-infected A/J mice. In light of this observation, the production of MIP-2 in the A/J mouse model was investigated so that the role of those chemokines

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induced during experimental Legionnaires' disease might be elucidated. MIP-2 has been described as the mouse equivalent of human IL-8, a C-X-C chemokine (159, 304). This molecule is a potent regulator of neutrophil activation (81). MIP-2 was detected in lung homogenates of A/J mice infected with live *L. pneumophila*; however, when mice were challenged with heat killed organisms MIP-2 was not detected. Because this model represents an infection that may spontaneously resolve, it is possible that a critical antigen dose of *L. pneumophila* may be important to stimulate alveolar macrophages to secrete cytokines. This secretion threshold was not achieved by injection of $1x10^6$ heat-killed *L. pneumophila*. Further studies are necessary to resolve these differences.

In agreement with the work of Brieland *et al.*, (59) data from this study suggested that IL-10 was not induced by *L. pneumophila* infection *in vivo*. This result may offer one reason why it is that A/J mice are eventually able to clear infection with sublethal inocula of *L. pneumophila*. Indeed, IL-10 has been shown to down-regulate and impair host defenses against a variety of pathogenic bacteria (118, 199, 147). In contrast, the presence of IL-12 has been shown to be critical to the induction of host resistance to intracellular pathogens and this may be a result of up-regulating the host cellular immune response (69, 129, 189, 343). In addition, it has been suggested that IL-12 plays a role in the host response to *L. pneumophila* (59). In this study, replication of *L. pneumophila* within the lungs of A/J mice stimulated the production of

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both p40 and p70 components of IL-12 and this cytokine was detected in BAL fluids, lung homogenates and sera. IL-12 p40 levels were 10-fold higher than the biologically active heterodimer IL-12 p70 in lung homogenates of infected mice. It has been documented that cells producing IL-12 *in vitro* or *in vivo* secrete a 10 to 100-fold excess of p40 over the biologically active p70 heterodimer (208, 321). Since the murine IL-12 p40 subunit can act as an IL-12 receptor antagonist by binding to the IL-12 β1 receptor subunit which results in blocking the binding of the IL-12 heterodimer, it may be that this represents an important process for controlling IL-12 activity. Because IL-12 can induce IFN-γ gene transcription, it has the capacity to induce cytokine production, predominantly IFN-γ (67, 205). In this study IL-12 (p40 and p70) production preceded the appearance of IFN-γ in BAL fluids and this suggested that IL-12 may be influencing the production of IFN-γ in A/J mice following *L. pneumophila* infection. IFN-γ was detected in lung homogenates and in sera of A/J mice challenged with *L. pneumophila*. The importance of IFN-γ has been addressed in numerous reports during infection with a number of intracellular pathogens (46, 84, 89, 128, 196, 231, 247, 332). Host resistance to intracellular bacterial pathogens depends on cell-mediated immunity and activation of macrophages by IFN-γ. The administration of recombinant IFN-γ was shown to protect mice against *L. monocytogenes* and lethal *M.*

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tuberculosis infection, while neutralization with anti-IFN-γ antibodies markedly exacerbated these diseases (71, 128). In addition, IFN-γ has been shown to inhibit *L. pneumophila* replication *in vitro* (31, 38, 65, 149, 249). IFN-γ in combination with other cytokines *in vivo* including, IL-12 and TNF-α, may serve to facilitate the elimination of *L. pneumophila* for mice and may aid in subsequent recovery.

This study demonstrated that *L. pneumophila* was capable of inducing the production of several important groups of cytokines in the lungs and sera of A/J mice during *L. pneumophila* infection. A better understanding of the immune mechanisms involved in host protection are of utmost importance so that the potential for alternative therapeutic strategies for Legionnaires' disease may be realized. These may include cytokine therapy and modulation.

This study provides new insight into the interaction of *L. pneumophila* with the host using the *in vivo* mouse model. This study further demonstrates the usefulness of the A/J mouse strain to study the pathogenesis as well as the host's specific immune response during *L. pneumophila* infection and should be an invaluable model to aide in elucidating the specific host-bacterial interactions during *L. pneumophila* infection. In addition, this model may be used to investigate alternative therapeutic strategies to combat Legionnaries' disease.

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CHAPTER VI

CONCLUSIONS

Legionnaires' disease is a severe and potentially fatal form of sporadic and epidemic pneumonia in humans. The causative agent, *Legionella pneumophila,* is a facultative intracellular bacterium that parasitizes alveolar macrophages and monocytes (188, 250). The organism is widely distributed in the environment and infection occurs through inhalation of contaminated aerosolized water droplets that descend into the lower respiratory tract leading to disease (90, 361). Attachment of this facultative intracellular pathogen to host cells forms the prelude to infection. Many such agents possess pili, fimbriae, capsular mucopolysaccharide, flagella, lectin-like molecules, and OMPs, all of which have been implicated as putative bacterial ashesins for parasitism of host cells (152, 24, 282, 123, 317). It has been demonstrated that complement receptors, CR1 and CR3 on human monocytes are one mechanism by which *L. pneumophila* mediates phagocytosis in an opsonin-dependent system (266). Bellinger-Kawahara *et al.* (26) demonstrated that complement component C3 fixes

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exclusively to the MOMP of *L. pneumophila* via the alternative pathway of complement activation. Recently an opsonin-independent mechanism has been proposed (155, 154) in which *L.pneumophila* enters cells in the absence of opsonic components; however, this mechanism is less well understood. Gibson *et al.* (154) demonstrated that complement receptors were not essential for *L. pneumophila* attachment to host cells. Indeed, blocking studies using monoclonal antibodies specific for CR1, CR3, CR4 or their subunits did not inhibit bacterial binding and did not prevent the intracellular replication of *L. pneumophila.*

In the first part of this study, the role of the MOMP as an adhesin for binding *L. pneumophila* to macrophage-like U-937 cells was assessed in an environment devoid of antibody and complement. Previous work in our laboratory by High *et al.* (170) involved cloning and sequencing the 25 kDa MOMP of *L. pneumophila.* The gene encoding the 25 kDa MOMP of *L. pneumophila* was transformed into *E. coli* JM 83 and the resultant *E. coli* LP 116 clone was shown to express the *Legionella*-MOMP in *E. coli* by Western blot analysis and immunofluorescence assay using both anti-*L. pneumophila* antiserum and *Legionella*-MOMP-specific MAb. The parent *E. coli* JM 83, and the genetically mutated *E. coli* JM 83 clone LP 116 were evaluated for attachment to macrophage-like U-937 cells. Comparison of *E. coli* JM 83 and LP 116 showed that the expression of the protein of the *Legionella*-MOMP gene facilitated organism binding to cells in the absence of opsonins. LP 116

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was 5 times more adherent than the parent *E. coli* strain by both VBCC counts and IFA. These results suggest that the MOMP of *L. pneumophila* was, in part, responsible for this opsonin-independent attachment to U-937 cells. As a facultative intracellular pathogen, *L. pneumophila* interacts with the cell membranes of alveolar macrophages prior to phagocytosis and, as a result, surface-located antigens are crucial to disease induction. It is postulated that an opsonin-independent mechanism of binding is central to the initial establishment of cell infection in the human lung. This is especially pertinent as it has been shown that the lung is essentially devoid of complement (277). The identification of the MOMP of *L. pneumophila* as an adhesive molecule which facilitates attachment of this organism to host cells may merit further investigation for the potential development of useful anti-adherence vaccines. Indeed, the use of this surface protein from *L. pneumophila* may prove useful for the prevention of Legionnaires' disease. Such vaccines could be of major importance for preventing infection and reducing mortality from Legionnaires' disease especially among high risk patients.

Resistance to and survival from legionellosis likely requires an active cellular immune response; however, the role of host immune responses in the pathogenesis of Legionnaires' disease is incompletely understood. In this study, a biphasic approach was taken, using both *in vitro* and *in vivo* models of experimental Legionnaires' disease to elucidate the production of various

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classes of cytokines during infection; thus, gaining a better understanding of the host response during *L. pneumophila* infection. Replication of *L. pneumophila* within adherent U-937 cells elicited the production of IL-1β and IL-8, while expressed levels of TNF-α, IL-12 and IFN-γ were not affected. Interestingly, U-937 cells challenged with *L. pneumophila* produced decreased levels of IL-10 as compared to uninfected controls. U-937 cells cultured with heat killed *Legionella* produced significantly greater levels of IL-8 and IL-10 as compared to live organism challenge. Heat kill of *L. pneumophila* may result in the unmasking of cryptic epitopes that may be responsible for this observed effect. In addition, formalin fixation which maintains the structure of the outer surface of bacteria, indicated that the molecule(s) responsible for the increased levels of IL-8 and IL-10 are located on the surface of the organism. Preliminary data suggests that epitope(s) on the surface of heat-killed organisms, are in part responsible for the increased levels of IL-8 and IL-10 secreted from U-937 cells. These results suggest that *L. pneumophila* organisms elicit specific cytokines in macrophage-like cells *in vitro*, and also downregulates the ability of these cells to produce specific cytokine types following infection.

A murine model of *L. pneumophila* lung infection was established by injecting *L. pneumophila* into the trachea of A/J mice. Following the acquisition of initial ED_{50} data, a sublethal infection was established. *L.*

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pneumophila actively replicated within lungs of A/J mice over time and produced pathologic and histopathologic evidence of the progression of disease. During pneumonic infection, *L. pneumophila* induced the production of the proinflammatory cytokines, IL-1β and TNF-α, of the immune-regulating cytokines, IL-12 and IFN-γ and of the chemokine MIP-2 as detected in BAL fluids, lung homogenates and the sera of mice. Viable organisms were necessary to stimulate a detectable host cytokine response, indicating that *L. pneumophila* modulated cytokine production in A/J mice by an as yet unknown mechanism. Animals were able to resolve infection indicated by clearance of *L. pneumophila* from the lungs of infected mice. The profile of cytokines produced during infection may have contributed to resolution of infection. IFN-γ and TNF-α have previously been shown to activate alveolar macrophages *in vitro* to inhibit the intracellular growth of *L. pneumophila* (249). In addition, IL-12 produced by monocytes, macrophages and neutrophils is required for a Th1 type (cell-mediated) host cytokine response. The cytokine profile produced in the A/J mice following *L. pneumophila* challenge would suggest the induction of a Th1 response indicated specifically by the production of IL-12 and IFN-γ with a paucity of IL-10. This type of host immune response should assist in resolving *L. pneumophila* infection as portrayed in the A/J mouse.

Alternatively, when the U-937 cell was used as a model of macrophage

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infection, there was no production of either IL-12 or IFN-γ. Although IL-10 was detected during *L. pneumophila* infection, lower levels were produced as compared to uninfected cells. In the *in vitro* system, U-937 cells were not able to combat infection and were eventually lysed. Striking differences were noted between *in vitro* and *in vivo* models and it is stressed that any comparisons between the two systems should be evaluated with care. It is our belief that the use of both systems provide invaluable information to yield a better understanding of the host response to *L. pneumophila* infection. Such studies may lead to the development of novel therapeutic strategies such as the use of cytokine therapy to assist in resolving clinical Legionnaires' disease. Recovery from legionellosis is associated with the development of cell-mediated immunity and patient susceptibility to Legionnaires' disease is linked to deficiencies in cellular immune responses. It is generally accepted that the failure to control or resolve infectious disease often results from inappropriate rather than insufficient immune responses. Therefore, development of a successful immune response with minimal pathology to an infectious agent is the desired goal of immunotherapy. The clinical implications of the findings from this study with regard to therapy or prophylaxis of Legionnaires' disease are manifold. Cytokines such as IL-12 and IFN-γ, that are important in the cell-mediated immune response may be a useful adjuvant in cytokine therapy, while exogenous administration of cytokines may enhance protective memory responses or compensate for the

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loss of a major cellular source of endogenous cytokines. In contrast, the use of cytokine antagonists active against immunosuppressive cytokines such as IL-10, could be helpful when endogenous, "inappropriate" cytokine expression sustains immunopathology and disease. In addition to these therapeutic perspectives, already supported by a considerable amount of data in experimental models of infection (199, 248, 292, 329), several possibilities can be envisioned that may carry the exploitation of the action of specific cytokines one step further than the simple administration or neutralization of specific cytokines. As noted during the present *in vitro* cytokine studies, microbial products located on the surface of the organisms are in part responsible for cytokine production. Studies by Blander and Horwitz have shown that the metalloprotease (43) and outer membrane material (41) elicit cellular immune responses in guinea pigs, thus, the MOMP, located on the surface of *L. pneumophila* may be, in part, responsible for the observed cytokine responses seen in the present study. A better understanding of the host response to *L. pneumophila* infection is central to the development of novel immunotherapeutic strategies. Furthermore, it would prove beneficial, to define those antigens of *L. pneumophila* that trigger protective cytokine production. Once the chemical nature of such microbial products has been established they could be molded artificially and possibly used as adjuvants for enhancing a potent *L. pneumophila* cell-mediated immune response. This approach has been used to test the ability of a recombinant

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adenovirus vector-expressing IL-12 to skew the immune response in a Th1 direction and prevent leishmaniasis, a disease caused by an intracellular protozoal pathogen, in susceptible BALB/c mice (140). These investigators suggested the potential use of recombinant adenoviruses expressing cytokines as potent immuno-modulatory agents for the generation of protective immune responses against intracellular pathogens. In addition, the potential vaccine properties of potentially protective antigens encoded for by genes in the *Mycobacterium tuberculosis* genome were evaluated for immunogenicity and protective efficacy against challenge with *M. tuberculosis* (331). Mice injected with plasmid DNA encoding proteins from *M. tuberculosis* resulted in significant levels of the Th1-type cytokines, IL-2 and IFN-γ. Mice "vaccinated" with DNA demonstrated significant and sustained reduction in bacterial CFU numbers in spleen and lungs. Thus, DNA vaccination may prove to be a powerful and easy method for comparative screening of potentially protective antigens from intracellular pathogens, such as *L. pneumophila*. The fine tuning and specific modulation of cytokines by cytokine therapy or by using potentially protective antigens for subunit vaccines, such as the MOMP, to provide an appropriate immune response may prove to be extremely beneficial to combating Legionnaires' disease.

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CHAPTER VII

FUTURE STUDIES

A multifaceted approach to investigating the interaction of *Legionella pneumophila* with the host both *in vitro* and *in vivo* was implemented in this study. The role of the MOMP as an adhesin for *L. pneumophila* attachment to host cells was evaluated *in vitro*. In addition, the complex cytokine host response during *L. pneumophila* infection was investigated using both *in vitro* and *in vivo* infection models. Throughout these studies many interesting lines of work developed which would merit further investigation

1. Isogenic mutants of *L. pneumophila* that do not express the MOMP could be constructed and evaluated in binding studies to elucidate attachment potential of these mutants to host cells. If these mutants are still able to bind to cells then additional bacterial adhesins may be involved in the complex event of organism attachment. *L. pneumophila* outer membrane preparations may be added to host cells in binding assays, and evaluated by gel electrophoresis to identify additional adhesins. In addition, the host cell receptor responsible for MOMP attachment should be defined. Once

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elucidated both adhesin and host cell receptor blocking studies could be executed as a preliminary step to developing anti-adhesin and/or anti-receptor vaccines to assist in controlling Legionnaires' disease.

2. To assess the role of the MOMP, in addition to other *L. pneumophila* antigens as immunostimulatory molecules capable of eliciting an immune response, purified MOMP or additional antigen structures may be used in both *in vitro* and *in vivo* assays. Initially, purified MOMP may be added to host cells and the cytokine response evaluated. Furthermore, the host cytokine response in mice may be defined following MOMP challenge. In addition, if the host response is protective, the possibility exists for using the MOMP or other antigens as adjuvants to develop a vaccine which is capable of mounting a protective immune response.

3. To address the role that specific cytokines play during *L. pneumophila* infection, several approaches may be implemented. 1) By using an effective dose $_{50}$ model in mice, various cytokines may be screened in both dose and time-dependent studies for their protective capabilities following *L. pneumophila* challenge. 2) Monoclonal antibodies can be administered to mice to deplete specific cytokines, that have shown protective abilities, prior to *L. pneumophila* infection. 3) Cytokine therapy can be employed by

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administering a particular cytokine to address pathology and outcome of infection during *L. pneumophila* challenge. To begin to address this point, A/J mice were administered either interferon-γ, IL-1β or IL-12, intraperitoneally prior to intratracheal challenge with 10^6 CFU of *L. pneumophila* to assess the ability of these cytokines to reduce bacterial numbers in mouse lungs.

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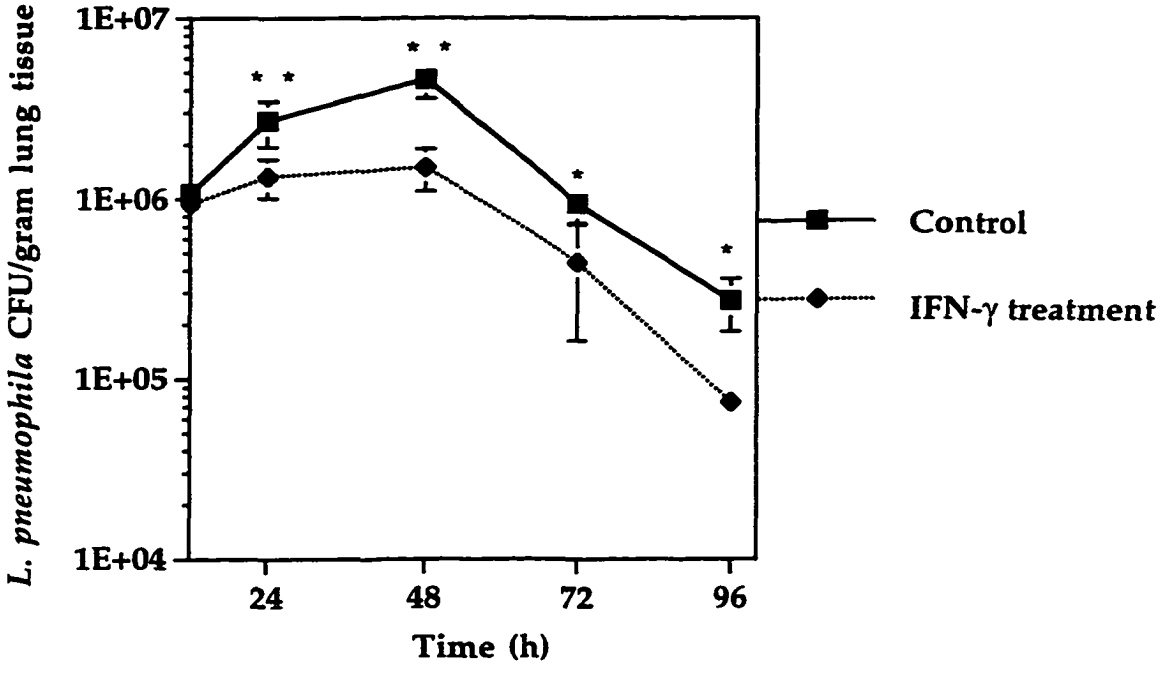

Figure 7.1. Exogenous cytokine treatment of A/J mice with 5 μg of interferon-γ. Recombinant IFN-γ was purchased from R & D systems and reconstituted as per manufacture's instructions. IFN-γ was administered intraperitoneally (0.1 ml) 24 h prior to *L. pneumophila* challenge. Animals were anesthetized and challenged intratracheally with 10^6 CFU of *L. pneumophila* as described in section 5.3.2. Animals were euthanized at specified time points and lungs were aseptically harvested, homogenized, serially 10-fold diluted and plated onto BCYE-PAC agar as described in section 5.3.4. *Legionella* colonies were enumerated after 72 h growth and the concentration of *L. pneumophila* was expressed as CFU per gram of lung tissue. The non-parametric Mann-Whitney test was performed to analyze differences between untreated and cytokine treated mice. Controls are mice that have been challenged with *L. pneumophila* and not pre-treated with cytokine. $^*p < 0.0014$, $^{**}p < 0.0004$.

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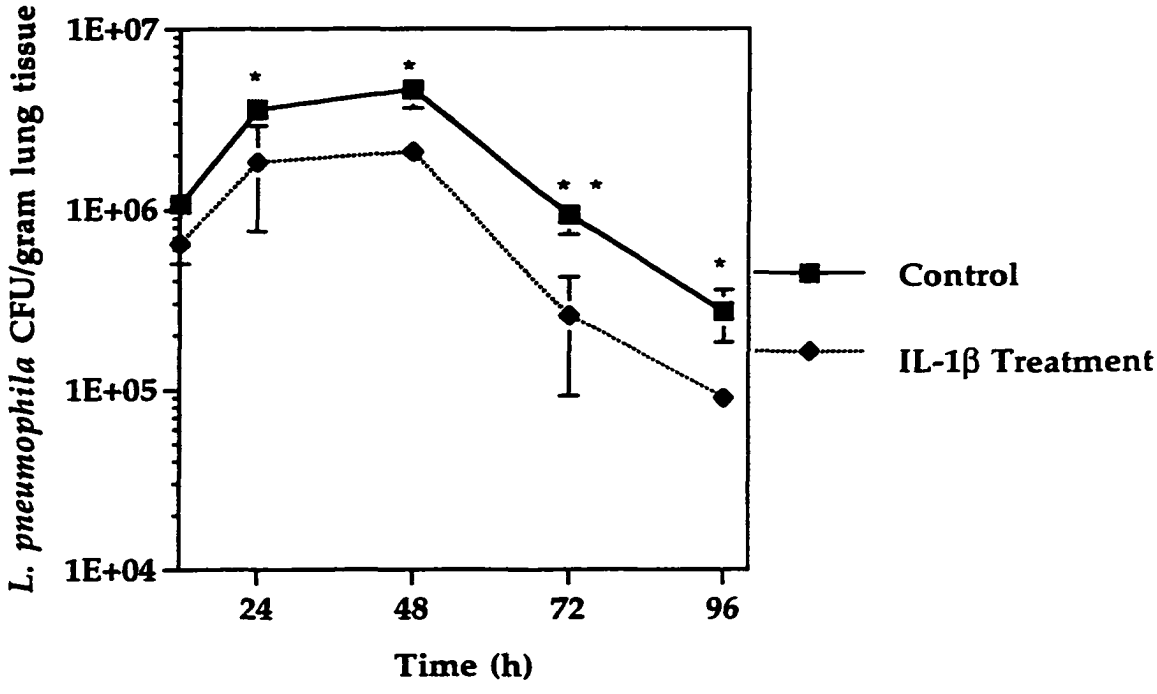

Figure 7.2. Exogenous cytokine treatment of A/J mice with 0.5 μg of interleukin-1β. Recombinant IL-1β was purchased from R & D systems and reconstituted as per manufacture's instructions. IL-1β was administered intraperitoneally (0.1 ml) 24 h prior to *L. pneumophila* challenge. Animals were anesthetized and challenged intratracheally with 10^6 CFU of *L. pneumophila* as described in section 5.3.2. Animals were euthanized at specified time points and lungs were aseptically harvested, homogenized, serially 10-fold diluted and plated onto BCYE-PAC agar as described in section 5.3.4. *Legionella* colonies were enumerated after 72 h growth and the concentration of *L. pneumophila* was expressed as CFU per gram of lung tissue. The non-parametric Mann-Whitney test was performed to analyze differences between untreated and cytokine treated mice. Controls are mice that have been challenged with *L. pneumophila* and not pre-treated with cytokine. $^*p < 0.03$, $^{**}p < 0.002$.

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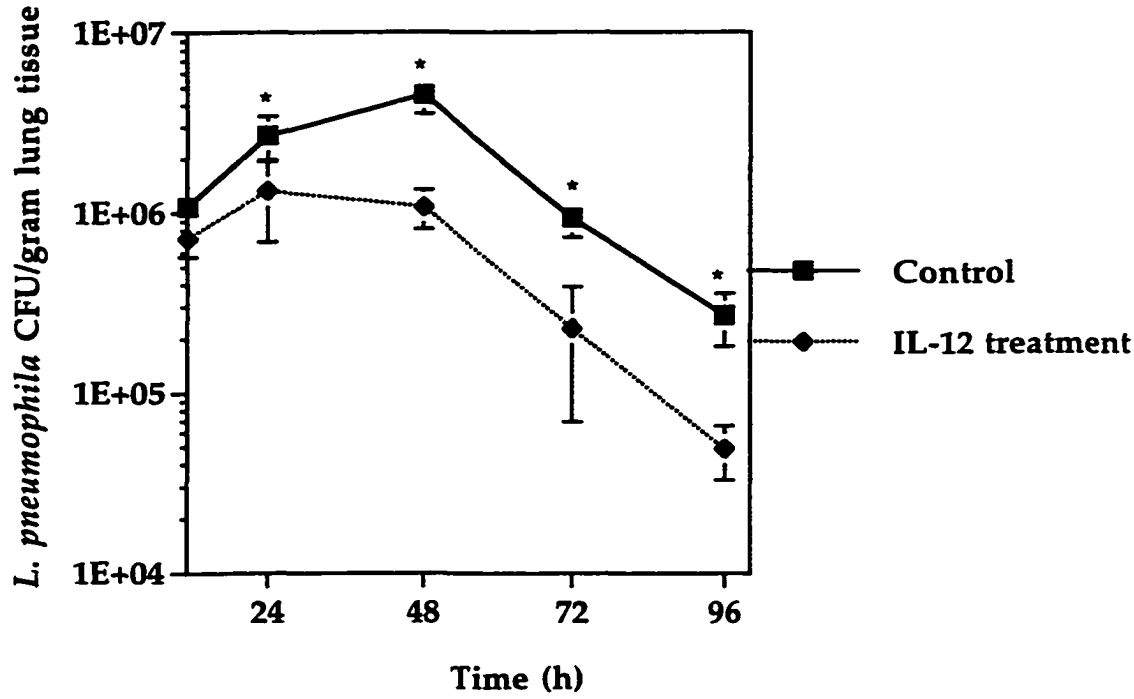

Figure 7.3. Exogenous cytokine treatment of A/J mice with 1 μg of interleukin-12. Recombinant IL-12 was purchased from R & D systems and reconstituted as per manufacture's instructions. IL-12 was administered intraperitoneally (0.1 ml) 24 h prior to *L. pneumophila* challenge. Animals were anesthetized and challenged intratracheally with 10^6 CFU of *L. pneumophila* as described in section 5.3.2. Animals were euthanized at specified time points and lungs were aseptically harvested, homogenized, serially 10-fold diluted and plated onto BCYE-PAC agar as described in section 5.3.4. *Legionella* colonies were enumerated after 72 h growth and the concentration of *L. pneumophila* was expressed as CFU per gram of lung tissue. The non-parametric Mann-Whitney test was performed to analyze differences between untreated and cytokine treated mice. Controls are mice that have been challenged with *L. pneumophila* and not pre-treated with cytokine. $^*p < 0.0059$.

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4. To elucidate the immunization capabilities of *L. pneumophila in vivo*, mice may be challenged with heat-killed organisms or purified antigens prior to infection with viable *L. pneumophila* and the ability of the host to resolve infection may be investigated. In addition, the host cytokine response may be determined and pathology studies conducted to further define the mechanism of protection.

5. Additional work is required to define the role of cell-mediated immunity and the part that T cells play during *L. pneumophila* infection. Mice may either be depleted of specific T cell subsets (CD4 or CD8) using monoclonal antibodies or transgenic mice devoid of a particular subset can be challenged with *L. pneumophila* to define the cytokine profiles during challenge. In addition, the pathology and outcome of infection could be evaluated under these conditions.

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APPENDICES

Appendix 1: Media used in biological

A. Bacterial cultivation

1.1. Storage of Bacterial Cultures. *Legionella pneumophila* serogroup 1 strain Nottingham 7 (N7) is a virulent clinical isolate from a fatal case of Legionnaires' disease. Bacteria were stored frozen in 1 % serum, 10 % sorbitol at -70°C throughout this study. Thawed aliquots were plated onto buffered charcoal yeast extract agar (BCYE-α) and grown at 37°C in a humid environment for 72 h.

Escherichia coli JM 83 and *E. coli* JM 83 clone LP 116 were stored frozen in 10 % glycerol at -70°C. Thawed aliquots were plated onto nutrient agar and grown at 37°C for 24 h. Frozen aliquots and all media used for *E. coli* JM 83 clone LP 116 were supplemented with 50 μg/ml ampicillin (Sigma, St. Louis, MO.).

1.2. Buffered charcoal yeast extract agar (BCYE-α). This media was purchased in dehydrated form from Becton Dickinson (Cockeyesville, MD.) and was resuspended in deionized water. The formulation of this agar is as follows:

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Component	Amount (gram/liter)
Yeast Extract	10.0
ACES Buffer	10.0
Ferric pyrophosphate	0.25
α-ketoglutarate	1.0
Agar	15.0
Activated Charcoal	2.0

37 g of BCYE-α was added to 800 ml of fresh deionized water and mixed until dissolved. The pH of the medium was adjusted to 6.9 by adding 10N KOH dropwise. The final volume was adjusted to 1 liter by adding deionized water. The agar was sterilized by autoclave using 121°C at 12 psi for 20 min and the media was allowed to cool to 52°C. Four ml of a 10 % L-cysteine solution in deionized water that was filter sterilized was aseptically added to each liter, evenly distributed throughout the media and poured into plates. Agar was allowed to solidify and plates were sterility checked in a 37°C incubator for 24 h and then stored at 4°C.

1.3. **Buffered Yeast Extract Broth (BYE-α).** BYE-α broth was used to grow *L. pneumophila* from plate grown cultures. This media was the standard broth for *L. pneumophila* cultivation and was not supplemented with antibiotics. The formulation was as follow:

Component	Amount (gram/liter)
Yeast Extract	10.0
ACES Buffer	10.0
Ferric pyrophosphate	0.25
α-ketoglutarate	1.0

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The above components were added to 800 ml deionized water. The pH of the broth was adjusted to 6.9 with the dropwise addition of 10N KOH and the total volume was adjusted to 1 liter with fresh deionized water. The BYE-α broth was filtered through a 0.45 μm filter and then through a sterile 0.22 μm filter. Four ml of a 10 % solution of L-cysteine in deionized water was aseptically added per liter and the broth was aliquot into 5 ml batches, sterility checked in a 37°C incubator for 24 h and then stored at 4°C.

1.4. **Nutrient agar or nutrient broth.** This media was purchased in dehydrated form from DIFCO laboratories (Detroit, MI) and was resuspended in deionized water. The formulation of this agar is as follows:

Component	Amount (gram/liter)
Beef extract	3.0
Peptone	5.0
(Nutrient agar only)Agar	15.0

23 g of dehydrated media was added to 800 ml deionized water. The total volume was adjusted to 1 liter with fresh deionized water. Both the agar and broth were sterilized by autoclave using 121°C at 12 psi for 20 min and the media was allowed to cool to 52°C. Agar was poured into plates and allowed to solidify and plates were sterility checked in a 37°C incubator for 24 h and then stored at 4°C. Broth was aliquot into 5 ml batches and sterility checked in a 37°C incubator for 24 h and then stored at 4°C. All media used to grow *E. coli* JM 83 clone LP116 contained 50 μg/ml ampicillin (Sigma).

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1.5. 1 % Peptone. 1 g of peptone (DIFCO) was dissolved in 100 ml of deionized water. The solution was sterilized by autoclaving (121°C at 12 psi for 20 min) and then aliquot in 0.9 ml volumes with a cornwall syringe into sterilized standard type dilution blank test tubes. These 0.9 ml dilutions were sterility checked and stored at 4°C.

B. Cell Culture

1.6. Hank's Balanced Salt Solution (HBSS) with or without Ca^{++} and Mg^{++}. Sterile batches of 500 ml 1X HBSS supplemented with phenol red were purchased from Cellgro (Cellgro, Hendon, VA.). Bottles were stored at 4°C until needed.

1.7. Fetal Bovine Serum. Sterile fetal bovine serum was purchased from Sigma in 500 ml batches. The contents of each bottle was thawed and aliquot into 50 ml batches in 50 ml sterile conical tubes and stored at -20°C until needed. FBS was heat inactivated by placing conical tube into a 56°C water bath for 30 min.

1.8. Sodium pyruvate, HEPES, NEAA and L-Glutamine. Above were purchased in sterile 100 ml batches (Cellgro). Sodium pyruvate was at a concentration of 100 mM (100 X) and was stored at 4°C. HEPES buffer was at a 1 M concentration and was stored at 4°C. NEAA was at 100 X and was stored

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at 4°C. L-Glutamine was at a concentration of 200 mM and stored at -20°C until needed.

1.9. RPMI-1640. U-937 cells and peritoneal macrophages from A/J mice were cultured in RPMI-1640 without antibiotics. Sterile batches of 500 ml 1X RPMI supplemented with phenol red was purchased from Cellgro. 70 ml was removed and the following components were added:

Component	Amount
Heat inactivated Fetal bovine serum (FBS)	50 ml
Sodium pyruvate	5 ml
HEPES	5 ml
*NEAA	5 ml
L-Glutamine	5 ml

* Non-essential amino acids

1.10. HAM's FK12. A549 cells were cultured in HAM's FK 12 medium without antibiotics. Sterile batches of 500 ml 1X HAM's FK12 supplemented with phenol red was purchased from Biofluids. 70 ml was removed and the following components were added:

Component	Amount
Heat inactivated Fetal bovine serum (FBS)	50 ml
Sodium pyruvate	5 ml
HEPES	5 ml
NEAA	5 ml
L-Glutamine	5 ml

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1.11. **Trypsin-EDTA.** Tryspin-EDTA was purchased as sterile 100 ml batches from Cellgro, aliquoted into 25 ml batches in sterile 50 ml conical tubes and stored frozen at -20°C.

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Appendix 2: Production of polyclonal antisera

2.1. Rabbits

White New Zealand rabbits were purchased from Charles River Laboratory and pre-bled to assess pre-immunization levels of antibodies against *L. pneumophila* and *E. coli* JM 83.

2.2. Antigen preparation

Whole cells of *L. pneumophila, E. coli* JM 83 and *E. coli* JM 83 clone LP 116 were grown on appropriate media (see Appendix 1.1) and formalin fixed for 1 h with 1 % formaldehyde (VWR) in 1X PBS to yield $1x10^9$ CFU/ml. Bacteria were washed 10 times with 1X PBS. Following the last wash, 1 ml bacterial suspensions were mixed with 1 ml Freund's incomplete adjuvant (VWR). Rabbits were subcutaneously administered 0.2 ml of the bacterial solutions on the right and left sides of their backs.

2.3. Bleeding procedure

2.3.1. Ear bleed. Rabbits used for both *L. pneumophila* and *E. coli* JM 83 were treated in the same manner. Samples of blood were aseptically obtained from the central artery of one of the rabbit's ears for antibody titer analysis following the immunization schedule. The rabbit's artery was vasodilated by warming the ear with a 15 watt bulb of a portable lamp. The ear was swabbed

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with alcohol and a 21-gauge needle was inserted into the artery to retrieve approximately 5 ml of blood into a sterile 15 ml conical tube. The ear was swabbed again with alcohol.

2.3.2. **Cardiac puncture.** Once the antibody titer had reached a suitable level, the final volume of blood was collected by cardiac puncture and exsanguination of the rabbit. An injection of Nembutal into the peripheral vein of the rabbit's ear was used to anesthetize the animal. Blood was drawn by inserting an 18-gauge needle through the chest into the heart. The rabbit was humanely exsanguinated with a final Nembutal injection into the heart. Blood collected was allowed to clot overnight at 4°C. Serum was collected by centrifugation at 300 x g and stored in 1 ml aliquots at -70°C. An antibody titer assay was performed on each rabbit.

2.4. Antibody titer assay

An indirect immunofluorescence assay was performed to assess titer of sera drawn from rabbits. *L. pneumophila* or *E. coli* JM 83 were fixed in 1 % formalin for 30 min., added to glass slides and allowed to dry. Sera were diluted in 1X PBS and added to the bacteria and incubated at 37°C for 1 h. Slides were washed three times with 1X PBS and goat anti-rabbit FITC conjugated serum was added to the bacteria on the glass slides and incubated at 37°C for 1 h. Slides were then washed three times with 1X PBS and viewed

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with a BH-2 Olympus microscope in the fluorescence mode. The most diluted serum sample from rabbits that exhibited a positive result was selected as the titer.

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Appendix 3: Reagents and Procedures used in Histological Studies

3.1. Harris' Alum Hematoxylin

The formulation of the solution was as follow:

Component	Amount
Hematoxylin crystals	5.0 g
Absolute alcohol	50.0 ml
Aluminum ammonium sulfate	100.0 g
Distilled water	1000.0 ml
Mercuric oxide	2.5 g

Hematoxylin crystals and the alum ammonium sulfate were dissolved in the alcohol and distilled water respectively by the aid of heat. The two solutions were removed from heat and mixed together and rapidly brought to a boil. Mercuric oxide was then added slowly and the mixture was reheated for 15-20 min. until a purple color was observed. The solution was quickly removed from heat and placed into a basin of cold water. The addition of 3.5 ml glacial acetic acid per 100 ml of solution increases the precision of the nuclear stain. The solution was filtered before use.

3.2. Stock 1 % Aqueous Eosin Solution

The formulation of the solution was as follow:

Component	Amount
Water soluble Eosin Y	5.0 g
Distilled water	500.0 ml
Glacial acetic acid	1.0 ml

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A working stock was made by adding 100 ml of the stock Eosin to 780 ml 95 % ethanol, 10 ml of a 1 % solution of aqueous phloxine B and 4 ml of glacial acetic acid.

3.3. Acid Alcohol

This was made by mixing 1000 ml of a 70 % alcohol solution (737 ml of 95 % alcohol with 263 ml distilled water) with 10 ml of concentrated hydrochloric acid.

3.4. Saturated Lithium Carbonate

This solution was made by adding 4.5 g of lithium carbonate was to 450 ml of distilled water.

3.5. Buffered formalin solution

A 10 % solution of formaldehyde (VWR) was made by adding 135 ml of a 37 % formaldehyde stock to 365 ml of 1X phosphate buffered saline (Cellgro). Aliquots of 40 ml were kept at room temperature.

3.6. Preparation of histological sections

A/J mice were euthanized by CO_2 (ACUC protocol # 960905) and the trachea isolated and tied. Lungs were perfused with 1 ml of a 10 % buffered formalin solution for 1 min. Lungs were excised and placed in 10 % buffered

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formalin until processed.

3.7. Histological processing of tissue samples

Lungs were cut and placed into embedding cassettes and washed overnight in water. Samples were placed into a Shandon Citadel Tissue Processor and the following cycle was used without the use of a vacuum or heat:

Treatment	Time
70 % ethanol	1.5 h
80 % ethanol	1.0 h
(95 % ethanol) X 2	1.0 h
(100 % ethanol) X 3	1.0 h
Histoclear	1.15 h
(Histoclear) X 2	1.5 h
Hot paraffin	2.15 h
Hot paraffin	2.5 h

Tissue samples were embedded in hot paraffin and allowed to harden at -20°C for 10 min. A facing knife was used to cut the excess paraffin and the tissues were then cut 6 μm thick using an American Optical Microtome. The sections were floated in a 45°C water that contained gelatin and allowed to drip dry. Samples were placed on a hot plate at 45°C for 15 min to dry and then in a 55°C incubator for 20 min. Samples were then stained with hematoxylin and eosin.

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3.8. Hematoxylin and eosin staining procedure

The following staining procedure was used:

Treatment	Time
(Histoclear) X 3	2 min.
(100 % ethanol) X 2	1 min.
100 % ethanol	2 min.
(95 % ethanol) X 2	1 min.
Distilled water	15 dips
Harris' Hematoxylin	12 min.
Running tap water	2 min.
Acid alcohol	3 dips
Distilled water	10 dips
Lithium carbonate	7 dips
Running tap water	10 min.
Eosin	5 min.
95 % ethanol	10 dips
(100 % ethanol) X 3	1 min.
(Histoclear) X 4	2 min.

3.9. Steiners' stain

3.9.1. Solutions

0.1 % uranyl nitrate. 0.05 g uranyl nitrate (BDH Chemicals Ltd, England) was dissolved in 30 ml distilled water.

0.1 % silver nitrate. 0.5 g silver nitrate (Fisher Scientific) was dissolved in 30 ml distilled water. Silver nitrate was preheated in a 70°C waterbath for 5 min. before use.

2.5 % gum mastic. 2.5 g gum mastic (MCB Manufacturing Chemists Inc., OH.) was dissolved in 100 ml absolute ethanol and filtered before use.

Hydroquinone. 0.25 g hydroquinone (Sigma) was dissolved in 25 ml distilled water.

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Developing solution. 15 ml gum mastic, 25 ml hydroquinone and 0.1 % silver nitrate were combined.

3.9.2. Procedure

Sections were deparaffinized, hydrated with distilled water and treated with 0.1 % uranyl nitrate. Sections were rinsed twice in distilled water and placed into 0.1 % preheated silver nitrate. Sections were rinsed twice in distilled water and dehydrated in 95 %, followed by absolute ethanol for 2 changes. Sections were treated with 2.5 % filtered gum mastic for 3 min., dried for 1 min. and then placed into preheated developing solution a 70°C waterbath with agitation for 10 min. Sections were rinsed in distilled water for 3 changes and dehydrated and cleared as follows:

Treatment	Time
95 % ethanol	10 dips
(100 % ethanol) X 3	1 min.
(Histoclear) X 4	2 min.

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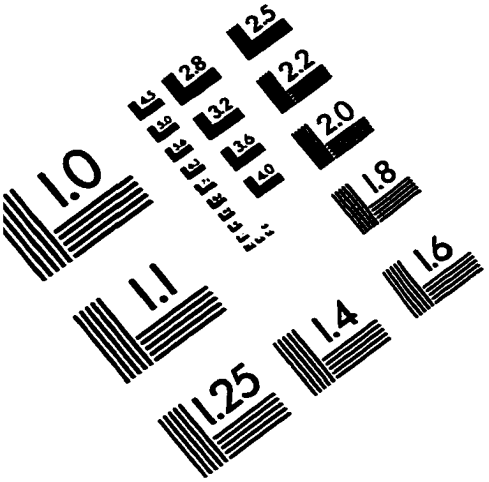
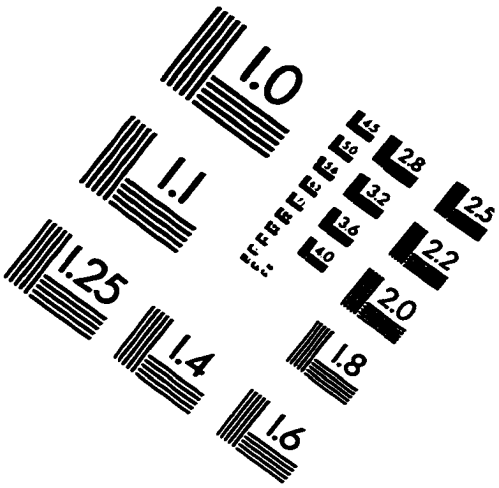
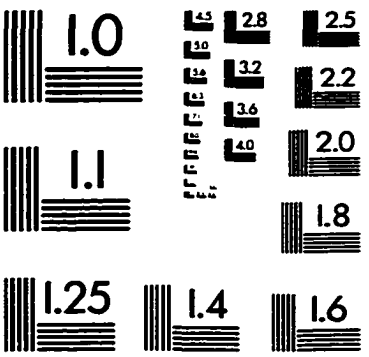
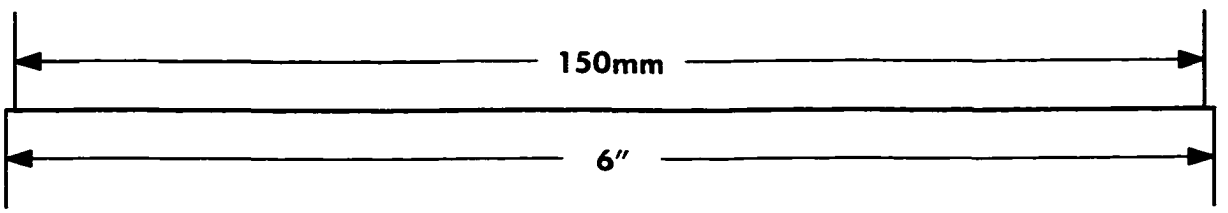
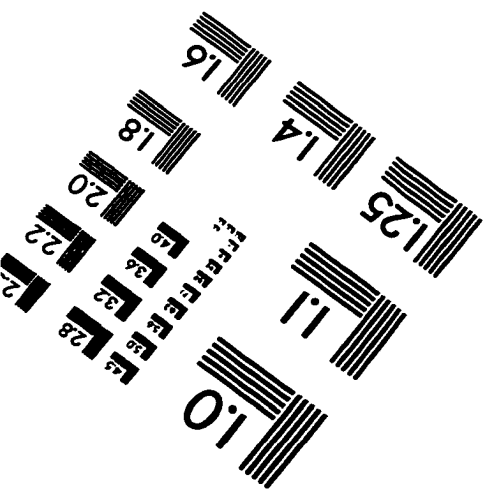
IMAGE EVALUATION
TEST TARGET (QA-3)











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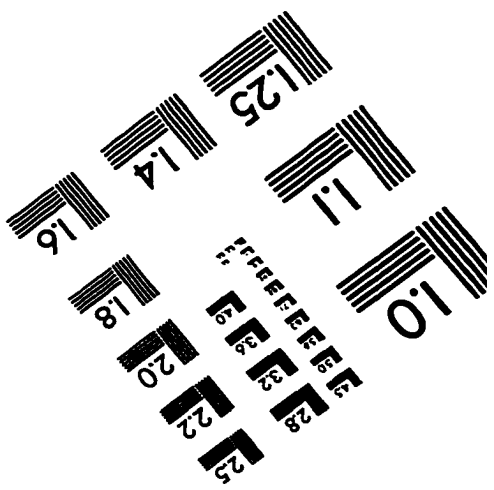

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